Medical Library

OCT 12 1942

VOL. XXIII. No. 4] [AUGUST, 1942

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

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136 GOWER ST., W.C. 1

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on behalf of

'The British Journal of Experimental Pathology.' Registered Office: 120 Moorgate, London, E.C. 2

Annual Subscription, January to December, post free, Two Pounds net
Single Numbers, Seven and Sixpence net

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

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THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XXIII AUGUST, 1942 No. 4

AN EXAMINATION OF THE O ANTIGENIC COMPLEX OF *BACT. TYPHOSUM.*

W. T. J. MORGAN AND S. M. PARTRIDGE.*

From the Biochemical Department, The Lister Institute of Preventive Medicine, London.

Received for publication May 20, 1942.

THE present study of the O antigen of *Bact typhosum* was undertaken at about the same time as an investigation on the nature of antigenic material isolated from *Bact. Shigae* by means of diethyleneglycol extraction (Morgan, 1937 ; Morgan and Partridge, 1940). In the latter work it was shown that the material extracted by diethyleneglycol, after suitable fractionation from aqueous solution with acetone, alcohol or ammonium sulphate, consisted essentially of a homogeneous complex which possessed the characteristic property of being readily dispersed in water to yield stable suspensions, the disperse phase of which consisted of polymolecular particles. The substance was shown to have the immunological properties of the dominant somatic antigen of Shiga's bacillus and on degradation yielded three main components, consisting of protein, phospholipin and specific polysaccharide. The phospholipin, although it was more or less firmly bound to the other constituents of the antigenic complex, could be removed without a significant change in the immunological properties of the antigen occurring. Finally, a means was found whereby the specific polysaccharide and protein components, themselves entirely without specific antigenic activity, could be recombined to form a powerful antigen possessing immunological properties similar to the parent antigenic complex (Partridge and Morgan, 1940).

In the present work, antigenic material extracted from *Bact. typhosum* (strain O 901) by a technique already described in detail (Morgan, 1937 ; Morgan and Partridge, 1940) has been resolved into its components, and has been found to behave in a strikingly similar manner to the antigen of *Bact. Shigae*. The antigenic material has again proved to be a complex of specific polysaccharide, phospholipin and protein, the latter being very similar in properties to the one contained in the antigenic complex derived from *Bact. Shigae*.

* Beit Memorial Research Fellow.

Unfortunately much of the work described in this paper, which was carried out during 1939 and 1940, still remains incomplete, and the results must therefore be considered preliminary in character. Publication is made at this stage because there seems little likelihood of repeating or extending the observations for some time to come.

EXPERIMENTAL.

Details of the production of mass cultures of *Bact. typhosum* and the extraction of antigenic material by means of diethyleneglycol have already appeared (Henderson and Morgan, 1938), and the technique described has been closely followed in the present work.

Primary Extraction Product.

The diethyleneglycol extraction product, after fractionation from 0·5–1·0 per cent. aqueous solution with ice-cold acetone, yielded a main fraction between the levels 33–50 per cent. acetone which, after solution in water and evaporation to dryness from the frozen state, gave an almost pure white fibrous product that readily dispersed in water to yield a heavily opalescent suspension.

The figures for elementary analysis of the primary extraction product were somewhat variable from preparation to preparation; N varied from 3·0–3·5 per cent. and the P figures between 1·6–1·8 per cent.

The product is powerfully antigenic in rabbits. A short course of immunization, consisting of three intravenous doses of 0·05 mg. at intervals of three days, gave sera that precipitated heavily with dilutions of the specific polysaccharide up to $1 : 10^6$, and which agglutinated completely formolized suspensions of *Bact. typhosum* (O 901) up to a dilution of 1 : 2000. The toxicity of the material for mice (M.L.D.) has already been determined as 0·1–0·2 mg. (Henderson and Morgan, 1938).

From 129 g. of dry bacteria (strain O 901) 7·4 g. of primary extraction product was obtained representing a yield of 5·7 per cent. Two preparations of "rough" *Bact. typhosum* ("rough" Ty 2) which possessed neither O Vi nor H antigen were extracted and fractionated as described above. From 53 g. and 85 g. of the dry organism, 0·39 g. and 0·42 g., representing 0·73 per cent. and 0·49 per cent. yield respectively, was obtained. This material has been taken as a measure of the amount of "rough" antigenic complex, of any non-specific somatic substances, or of adsorbed agar and products of bacterial metabolism that could be present in the O antigenic preparations. The amount is equivalent to about 10 per cent. of the O antigenic complex.

Acid Hydrolysis.

The dry O antigenic material was thoroughly extracted with chloroform and pentane in order to insure that no uncombined lipoid remained in the preparation. The defatted material was then suspended in water to yield a 1 per cent. solution. When left undisturbed in the cold room for several days the suspension showed signs of sedimentation. Acetic acid was added to the

suspension to yield a concentration of 1 per cent. and the mixture was heated at 97–99° for four hours. One hour's heating caused a flocculant precipitate to separate from the suspension and the supernatant solution rapidly became clear on further heating.

Ether-soluble component (*fraction I*).—After cooling the mixture was extracted with ether, the ether extract was washed with a little dilute alkali, then with a few ml. water and dried over anhydrous Na_2SO_4. The residue, after removing the ether, was found to be almost completely soluble in pentane. The pentane solution after evaporation to dryness gave a pale yellow semi-solid substance. The material was not soluble in alcohol, showed $[\alpha]_{5461}$ + 8° ± 2° (in pentane) and contained 3·2 per cent. organically bound P and 1·2 per cent. N. The N : P ratio was 1 : 2·7, which indicated that the fatty material most probably belonged to the monoaminophospholipin group of substances, and resembled the corresponding material isolated from the antigenic complex of *Bact. Shigae*. The pentane-soluble substances represented 5–7 per cent. of the whole antigenic material in different preparations, and by analogy with the material found in the antigenic complex of *Bact. Shigae* has been termed " phospholipin." The material has not been examined further.

Acid-insoluble component (*fraction II*).—The crude water-insoluble substance remaining after ether extraction represented about 25 per cent. of the antigenic complex and, like the corresponding fraction isolated from *Bact. Shigae*, was soluble in dilute alkali but insoluble in dilute acid. After purification, by repeated dissolution in dilute alkali to pH 8·5 and reprecipitation with dilute acetic acid, the substance contained 11·5 per cent. N, and the weight of material finally recovered represented about 20 per cent. of the total antigen. The material gave all the usual qualitative tests for proteins.

Water-soluble component (*fraction III*).—The clear supernatant fluid after the removal of fractions I and II contained the specific polysaccharide. This was recovered by evaporating the solution to a small volume, dialysing, to remove material of low molecular weight, and precipitating the material by addition of alcohol to yield 80 per cent. by volume. The crude substance so obtained represented, in different preparations of the antigenic material, 50–55 per cent. of the primary extraction product used. The figures for the polysaccharide yield, however, must be considered low owing to the appreciable solubility of the crude material in aqueous alcohol. As will be shown later, the product is changed during the hydrolysis with acetic acid, and is therefore best described as " degraded polysaccharide."

The Degraded Polysaccharide Component of the Antigenic Complex.

The crude polysaccharide preparations, obtained by 1 per cent. acid hydrolysis of the primary diethyleneglycol extraction product, contained 1·0–1·3 per cent. N, 1·0–1·2 per cent. P, and showed $[\alpha]_{5461}$ + 93° ± 5° (c, 1 per cent. in H_2O). The material gave a positive test for arginine and was not completely soluble in water. The substance was therefore triturated with sufficient water to make a 5 per cent. solution, the insoluble material was removed and the clear supernatant was precipitated by the addition of 15 times its volume of alcohol; the polysaccharide was collected after standing overnight at 0°.

This treatment was repeated three times, after which procedure the material readily yielded a clear 10 per cent. aqueous solution, but still gave rise to a trace of pink colour when tested for arginine by Sakaguchi's reaction. The preparation was again dissolved in water to yield a 10 per cent. solution and precipitated by addition of ten times its volume of glacial acetic acid. The precipitate was recovered by centrifugation, washed thoroughly with alcohol and dried. The polysaccharide now gave a negative test for arginine. A further quantity of the material was recovered by the addition of glacial acetic acid to yield 96 per cent. in the final solution, but the material precipitated under these conditions gave strong Sakaguchi and biuret tests and was obviously contaminated with amino-acid containing material. The purified polysaccharide contained 1·2 per cent. P in organic combination, no inorganic P, less than 0·1 per cent. N, and showed $[\alpha]_{5461} + 128°$ (c, 1·0 per cent. in water). It gave rise to distinct precipitation to a dilution of $1 : 2 \times 10^6$ when mixed with *Bact. typhosum* O immune rabbit serum. The material (2 mg.) given intravenously was non-toxic for mice. On treatment with 0·5 *N* sulphuric acid at 100° the polysaccharide yielded a maximum of 48 per cent. reducing sugars calculated as glucose (Shaffer-Hartmann copper reduction method). Hydrolysis in stronger acid was not investigated. A 1·0 per cent. solution of the polysaccharide in 1 per cent. NaCl solution gave a relative viscosity of 1·17 (solvent = 1) when measured at 37° with a modified Ostwald viscosimeter. The polysaccharide, like similar preparations from other organisms, did not have the power to induce the formation of specific O precipitins or agglutinins in rabbits. The polysaccharide has not been exhaustively fractionated from solution under different conditions and with different solvents, and it is therefore not possible to claim that the properties of the material described are those of the pure specific polysaccharide hapten of the O antigen of *Bact. typhosum*.

The Protein Component.

The water and ether-insoluble product (fraction II) from the 1 per cent. acetic acid hydrolysis of the antigenic complex contained after purification 11·5 per cent. N (Dumas), 0·47 per cent. P, showed a laevo rotation $[\alpha]_{5461} - 55° \pm 5°$ and gave a strongly positive colour reaction for arginine and tyrosine. Like the product obtained from *Bact. Shigae*, it is insoluble in an excess of mineral acid, but is soluble in dilute alkali and in phosphate buffer at pH 8·5. Through the kindness of Dr. R. A. Morton we have been able to compare, under the same conditions, the ultraviolet absorption spectra of the protein of *Bact. typhosum* with the corresponding component of the antigen of *Bact. Shigae*. In Fig. 1 the values for $E\ ^{1\ \text{per cent.}}_{1\ \text{cm.}}$ (log. Io/I for a 1 cm. layer of a 1·0 per cent. concentration in 0·1 *N* NaOH) are plotted against the wave-length of the light adsorbed in millimicrons. Preparations of the protein component obtained by acid hydrolysis of the O antigens of either organism are usually contaminated with a yellowish pigment which is difficult to remove completely, and which induces a source of error in the ultraviolet absorption measurements. Some correction for absorption due to this pigment can be made when using the curves for the purpose of calculating the tyrosine and tryptophane content

of the protein according to the method of Holiday (1936), as neither of these amino acids show any significant absorption at wave-lengths greater than 340mμ., and the part of the curve at greater wave-lengths than this may be extrapolated to afford correction values. Using the equations given by

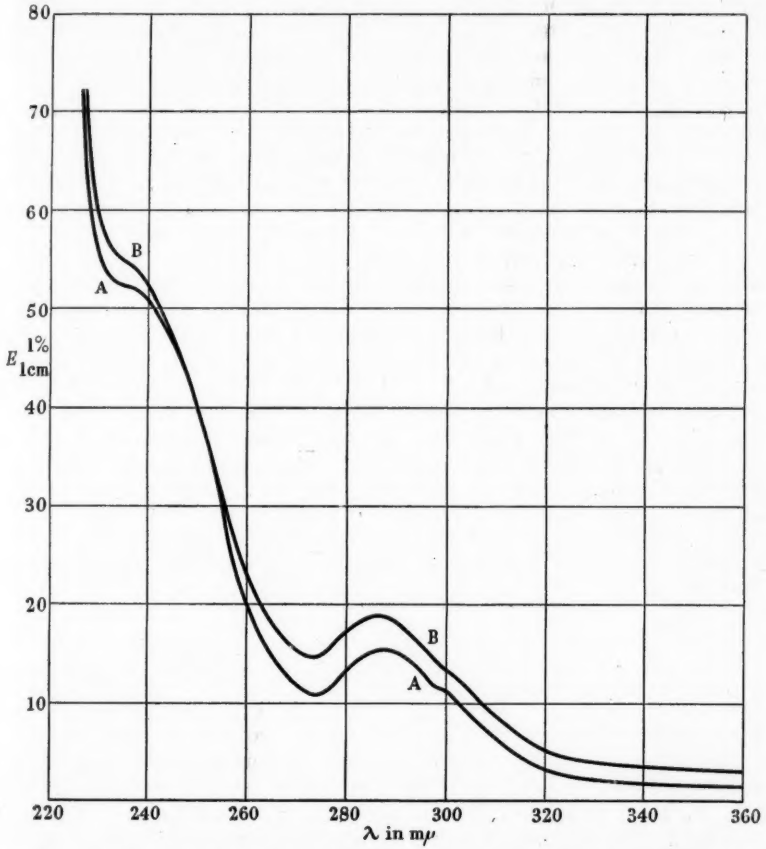

FIG. 1.—Showing the ultra-violet absorption spectra of the protein components of the O antigenic complex of (A) *Bact. typhosum*, (B) *Bact. Shigae* in 0·1 *N* NaOH.

Holiday (1936) the tyrosine content of the *Bact. typhosum* O protein component is calculated at 6·5 per cent. This figure shows close agreement with that for the tyrosine content of the protein component of the O antigen of *Bact. Shigae*, and indeed, if the greater correction for the yellow colour of the solution of the " Shiga " protein is allowed for, it will be seen that the two absorption curves are almost identical.

It will be observed that both curves show a marked inflection at 230–245 mμ. This is an unusual feature in the absorption spectrum of a protein, and

it is possible that the absorption in this region is associated with a prosthetic group which may be common to the protein components of both antigens.

A relationship between the two protein components may also be demonstrated by immunological methods. It has already been shown (Morgan and Partridge, 1940, 1941) that the corresponding protein material isolated from the antigen of *Bact. Shigae* induces the formation of homologous precipitins when injected into rabbits. Indeed, when precautions are taken to avoid the use of strong alkali during its preparation the protein proved to be a good antigen. The corresponding material isolated from *Bact. typhosum* was also found to be active in producing homologous precipitins when injected into rabbits. Two rabbits received eight intravenous doses, each of 0·05 mg. of the protein component, at three-day intervals and were bled five days after. In each instance the immune serum reacted to an equal degree with both homologous and heterologous proteins, marked precipitation being observed with an antigen dilution of $1 : 10^5$.

Additional evidence which again indicates the close similarity of the two substances was obtained in anaphylaxis experiments. Six guinea-pigs were passively immunized with an intraperitoneal dose of *Bact. typhosum* protein immune rabbit serum. After an interval of 24 hours the guinea-pigs were divided into two groups and half were given an intravenous dose (5 mg.) of the protein derived from the O antigen of *Bact. typhosum* and half a similar dose of " Shiga " protein. Both groups of animals showed definite signs of anaphylactic shock, but the shock produced was in no case fatal. The animals recovered, except for extreme exhaustion, after about fifteen minutes.

It has been shown elsewhere (Morgan and Partridge, 1941*a*, *b*) that the protein component isolated from the O antigen of *Bact. Shiga* by acid hydrolysis is probably a conjugated protein. The close similarity in chemical and immunological properties of the protein component of the O antigen of *Bact. typhosum* to that of the corresponding " Shiga " component indicates that the *Bact. typhosum* substance may also be a conjugated protein. The term " conjugated protein " will be used to describe the protein component and to distinguish it from the simple protein, to be described later in this paper, that is derived from it by the action of phenol or of alkali.

Partial Degradation of the Antigenic Complex.

The primary diethylene glycol extraction product of *Bact. typhosum* dissolves readily in pure formamide. After careful fractionation of the formamide solution by the addition of cold ethyl alcohol a product is obtained which is free from the phospholipin. The diethyleneglycol extracted material (1·1 gm.) was dissolved in formamide (75 ml.) and allowed to stand overnight at 2–4° C. Ice-cold ethyl alcohol (225 ml.) was rapidly added with stirring and the precipitate was removed by centrifugation. The precipitate was redissolved in formamide (50 ml.) and again precipitated, this time by the addition of 100 ml. alcohol. A third precipitation from formamide solution at the 66 per cent. alcohol level was made and the product (0·55 gm.) was recovered after solution in water, dialysis to remove the remaining organic solvent and drying *in vacuo* from the frozen state.

The phospholipin-free antigen.—The substance contained 4·62 per cent. N and 1·2 per cent. P, yielded a heavily opalescent solution in water, but, unlike the primary extraction product, the solution is immediately rendered clear and colourless by the addition of dilute NaOH with subsequent adjustment to give pH 8·0. On hydrolysis with 1 per cent. acetic acid the material behaved in a manner similar to that of the primary extraction product, but no phospholipin could be recovered from the products of hydrolysis. In one experiment about 46 per cent. of the antigenic material was recovered as crude polysaccharide and 38 per cent. as crude protein. The high content of the protein component in this preparation of phospholipin-free material was due to the partial loss of specific polysaccharide from the antigenic complex during the thorough formamide treatment. The phospholipin-free complex is powerfully antigenic in rabbits; six doses of 0·05 mg. at intervals of three days yielded sera which precipitated with the specific polysaccharide and agglutinated formolized suspensions of *Bact. typhosum* (O 901) in serum dilutions as high as 1 : 20,000. The results of toxicity determinations in mice with one preparation showed the M.L.D. to be about 0·5 mg. Observations were made for 72 hours after the dose had been injected intravenously in 0·85 per cent. NaCl solution.

Action of trypsin.—Unlike the polysaccharide-protein complex isolated from *Bact. Shigae*, the phospholipin-free O antigen of *Bact. typhosum* could not be rendered free from nitrogenous components by tryptic digestion. The phospholipin-free antigen (400 mg.; N, 4·62 per cent.) was dissolved in water (40 ml.), 0·2 *M* Na_2HPO_4 solution (5 ml.) was added, and the mixture was incubated at 37° for 20 hours in the presence of chloroform with successive small additions of commercial trypsin. The heavily opalescent solution became quite clear two or three minutes after the first addition of trypsin. A total of 30 mg. of trypsin was used. The clear solution was dialysed for two or three days against distilled water and the volume of the solution was reduced to about 4 ml. by evaporation under reduced pressure. The material was precipitated from solution by addition of three times the volume of alcohol, and the precipitate was collected and dissolved in anhydrous formamide (20 ml.). After standing at 0–4° C. overnight the mixture was fractionated by the addition of ice-cold alcohol. A first precipitate was collected up to 40 per cent. alcohol and contained the bulk of the polysaccharide. A second, smaller precipitate appeared between the alcohol levels 40 and 66 per cent., but this, on examination, was found to be heavily contaminated with nitrogenous material, presumably trypsin and associated impurities and was not further examined. The main fraction amounted to about 150 mg., and after dialysis for 4–5 days to remove formamide, contained 1·56 per cent. N and 1·9 per cent. P. The product, as freshly prepared by tryptic digestion, yielded a perfectly clear solution in water, but subsequent treatment with organic solvents rendered it less readily soluble, and the material after swelling redissolved in water to yield solutions which were similar in appearance to solutions of starch. The relative viscosity of a 1 per cent. solution in 1 per cent. NaCl at 37° (as determined in a modified Ostwald viscosimeter) was 1·95 (solvent = 1). A polysaccharide hapten isolated from the antigenic complex of *Bact. Shigae* by this technique possessed a considerably higher viscosity, and it is of interest that the viscosity of the O polysaccharide should be so low.

The action of phenol.—Attempts were made to remove the nitrogenous components of the antigenic complex by shaking strong (2–3 per cent.) aqueous solutions of the antigenic material with an equal volume of 95 per cent. phenol solution. The mixed solution separated into two layers on standing, an aqueous layer and a lower phenol layer. Both layers were separated and thoroughly extracted with ether to remove phenol and the material remaining in each layer was examined. A sharp separation of the antigenic complex into a water-soluble polysaccharide and a phenol-soluble protein component was not attained, but the complex was deprived of its phospholipin component during the phenol treatment. Antigenic material isolated after phenol treatment showed a lower N content than the primary diethyleneglycol extraction product. The N varied from 2·0–2·5 per cent. in different preparations, whereas the organically bound phosphorus remained within the range 1·7–2·0 per cent. The antigenic material isolated by Palmer and Gerlough (1940) showed a considerably higher nitrogen content which varied from 3·4 to 3·6 per cent. In view of Palmer and Gerlough's results a preparation of O antigenic material (670 mg.) was extracted with 90 per cent. phenol solution (30 ml.). The first phenol supernatant gave a positive biuret test, whereas the two following extracts, each of 30 ml. 90 per cent. phenol solution, gave only a faintly positive test. The material remaining insoluble in 90 per cent. phenol solution was treated with alcohol to remove phenol and dried *in vacuo*. The substance contained 1·76 per cent. N and 1·87 per cent. organically bound P, was partly soluble in warm water and yielded an opalescent aqueous solution. The biuret test was weak but definitely positive on 10 mg. of the substance, and 2·5 mg. of the material gave a positive Sakaguchi test equal in intensity to 0·015 mg. arginine. The material was precipitated from aqueous solution by alcohol, dried and again treated with 90 per cent. phenol solution. The phenol-insoluble material finally obtained was separated into water-insoluble and water-soluble components. Both fractions appeared to be similar in composition. The water-soluble portion contained 1·74 per cent. N and 1·92 per cent. P, whereas the fraction sparingly soluble in water contained 1·77 per cent. N and 1·94 per cent. P. The preparations were free from inorganic P. The water-soluble material was precipitated from solution by alcohol, and, after drying, again treated with phenol. This time, however, the treatment of the phenol insoluble fraction with water gave rise to only a small amount of water-insoluble material. The water-soluble substance now contained 1·77 per cent. N, 1·60 per cent. organically bound P and again gave a definite Sakaguchi test. A quantitative estimation (Jorpes and Thorén, 1932) showed that 2·5 mg. of the preparation gave a colour equivalent to 0·015 mg. arginine. The ninhydrin reaction was negative. The substance still possessed antigenic properties, and three doses each of 0·001 mg. given intravenously to 5 rabbits gave rise to specific O agglutinins the titre of the sera varying from 1 : 1000 to 1 : 10,000. A 1 per cent. solution of the substance was somewhat opalescent, and on heating with 0·1 *N* acetic acid disaggregated in a manner similar to that already described for the diethyleneglycol primary extraction product. After 4 hours at 97–99° a flocculent precipitate was formed and the solution became clear. The precipitate was readily soluble in dilute NaOH, and was again thrown out of solution on the

addition of dilute acetic acid to pH 5·0. From these experimental results it appears that the phenol-treated antigenic complex contains about 12 per cent. of the protein component.

The action of dilute alkali.—The experiments described above illustrate the difficulty of removing the protein component of the antigen even when reagents which readily dissociate the antigenic complex of *Bact. Shigae* are used. In the presence of dilute NaOH, however, the antigenic complex is to a considerable extent dissociated. A sample (400 mg.) of the phospholipin-free antigen (4·62 per cent. N) was dissolved in 0·02 *N* NaOH (25 ml.). A clear solution was obtained, and after standing at room temperature for 10 minutes, was treated with ice-cold alcohol (50 ml.) and the precipitate formed was collected. The process of solution in dilute alkali and reprecipitation with alcohol was repeated 4 times, after which procedure the final precipitate was washed with absolute alcohol and dried *in vacuo*. The product was largely polysaccharide in nature, formed viscous, slightly opalescent solutions (2 per cent.) in water and contained 1·2 per cent. N. The material (200 mg.) was dissolved in water and dried from the frozen state. It was then extracted, first with 10 ml. 90 per cent. phenol solution, followed by two extractions with 80 per cent. phenol. The phenol was removed by repeated washing with ethanol and the material was dissolved in anhydrous formamide (10 ml.). The clear formamide solution was treated with 2 volumes of alcohol, the material thrown out of solution was washed with alcohol to remove formamide, and dialysed against several changes of distilled water. The substance was finally recovered after drying from the frozen state, and was found to dissolve in water to yield a solution which showed a faint bluish opalescence both at acid and at alkaline reaction. The substance contained 0·57 per cent. N, 1·62 per cent. P, and gave a precipitation reaction with rabbit O immune serum comparable to that given by the purified, degraded polysaccharide already described.

It will be seen from the above experiment that during the four precipitations with ethanol from alkaline solution the nitrogen content of the antigenic complex fell from 4·62 per cent. to 1·2 per cent., thus showing that during the process a large part of the protein component had been removed. In order to recover this protein the alcoholic NaOH supernatant solutions from the first two precipitations were mixed and acetic acid was added to give pH 4. A precipitate formed, was collected, and was found to dissolve in dilute NaOH to yield a clear solution. The substance was quantitatively recovered from the NaOH solution by addition of acetic acid to pH 5, but it was found to possess an appreciable solubility in *N* acetic acid yielding an opalescent solution which withstood prolonged centrifugation at 3000 r.p.m. in the laboratory angle centrifuge. Recovery from solution in *N* acetic acid was effected by raising the pH to 5 by addition of NaOH when a heavy flocculent precipitate was formed. The substance (40 mg.) gave a strong biuret test, and was freed from contaminating polysaccharide by repeated precipitation at pH 4·5 from slightly alkaline solution. The crude protein so obtained contained 13·9 per cent. N, 0·25 per cent. P and showed $[\alpha]_{5461} - 58° \pm 4°$. We have had no opportunity of examining this material in greater detail.

The Antigenicity of Undegraded Polysaccharide.

Although the degraded polysaccharide prepared by dilute acetic hydrolysis of the primary extraction product may be obtained free from nitrogen by simple purification methods, it will be observed that in the case of the undegraded polysaccharide the prolonged purification procedure outlined in the foregoing section yielded a product that still contained 0·57 per cent. N. It is not known whether the remaining nitrogen is due to a residue (4–5 per cent.) of intact conjugated protein, but animal experiments show that during the progressive removal of the protein component the antigenicity of the complex is considerably diminished, especially with respect to its capacity to produce precipitins against the purified polysaccharide. A group of 4 rabbits were

TABLE I.—*Showing the Production of* O *Precipitins and Agglutinins in Rabbits Immunized Intravenously with Six Doses of* (a) *the Artificial Antigenic Complex, and* (b) *the Undegraded Specific Polysaccharide.*

Nature of material injected.	Dose. (mg.).	Rabbit No.	Precipitation with specific polysaccharide from strain O 901. Dilution of hapten. 1 : $10^{\frac{1}{2}}$.	1 : 10^{1}.	1 : $10^{1\frac{1}{2}}$.	Agglutination of formolized *Bact. typhosum* (strain O 901). Dilution of serum. 1 : 100.	1 : 200.	1 : 500.	1 : 1000.	1 : 2000.
Artificial complex containing undegraded O polysaccharide and conjugated protein from *Bact. Shigae*	0·05	235	3	2	1	4	4	4	2	1
		243	3	2	1	4	4	4	4	2
		257	2	3	1	4	4	4	3	2
O 901 undegraded specific polysaccharide (N, 0·57 per cent.	0·05	272	0	0	0	4	2	1	0	0
		301	0	0	0	4	3	2	1	0
		305	0	0	0	4	3	1	0	0
		307	0	0	0	4	4	4	2	0

4, complete agglutination ; 3, heavy precipitation or almost complete agglutination ; 2, definite precipitation or agglutination ; 1, trace of precipitation or agglutination ; 0, no precipitation or agglutination.

injected with 6 doses of the undegraded polysaccharide (Table I). The sera gave no precipitin reaction with the polysaccharide, but the more sensitive agglutination test showed that the substance was nevertheless antigenic and that O agglutinins were present in the sera. In a parallel experiment the undegraded polysaccharide (N, 0·57 per cent.) preparation was combined with a sample of conjugated protein isolated from *Bact. Shigae*, by mixing alkaline solutions of the two substances and acidifying to pH 4–5 with acetic acid according to a method already described (Morgan and Partridge, 1941*b*). The artificial complex was injected into a second group of rabbits as shown in Table I. All the animals responded with the production of strong O precipitins and agglutinins.

DISCUSSION.

It has already been demonstrated that the product obtained by extracting *Bact. typhosum* (strain O 901) with anhydrous diethylene glycol exhibits the immunological behaviour of the O antigen (Henderson and Morgan,

1938). Some evidence was given to show that the material was largely homogeneous, and that it possessed a number of characteristic physical and chemical properties. The antigenic material has now been prepared in larger quantities, and the chemical nature of the product has been further examined along lines similar to those reported in the work on *Bact. Shigae* (Morgan and Partridge, 1940).

There are certain points of similarity between the basic chemical structure of the two antigenic preparations as isolated by means of diethyleneglycol extraction. Both products consist of a molecular aggregate that contains polysaccharide, phospholipin and a protein complex. By the action of anhydrous formamide both antigenic materials are readily freed from the phospholipin component without any appreciable loss in antigenicity.

In the course of the work on the O antigen of *Bact. typhosum* it soon became evident that the amino-acid complex (conjugated protein component) of the primary extraction product was indistinguishable in many of its properties from that derived from the somatic antigen of *Bact. Shigae*. A general comparison of certain of the properties of the two materials was therefore made. Dr. R. A. Morton kindly compared the ultraviolet absorption spectra of the two components under the same conditions and the resulting curves exhibited close similarity. The protein material from either source was antigenic when tested in rabbits, and the sera produced against the protein isolated from the typhoid antigen precipitated equally well with the conjugated protein material prepared from *Bact. Shigae*. Furthermore, guinea-pigs immunized with typhoid protein immune rabbit serum suffered anaphylactic shock when given either protein intravenously. It was also possible to replace the "Shiga" conjugated protein with that isolated from the O antigen of *Bact. typhosum* in the reconstitution of the "Shiga" antigen by the methods already described (Partridge and Morgan, 1940). It appears therefore that the protein complexes isolated from the two antigenic preparations are very similar in chemical and immunological properties. It may be recorded in passing that since this work was completed we have isolated from the specific antigenic complex of *Bact. dysenteriae* (Flexner) VI, strain 88 (Boyd, 1940), a protein component, $[\alpha]_{5461} - 50° \pm 5°$; N, 10·7; P, 1·1 per cent., similar in all the properties examined to the conjugated protein components derived from the O antigens of *Bact. Shigae* and *Bact. typhosum*.

An attempt was made to remove the conjugated protein component from the antigenic complex by means of digestion with trypsin, but unlike the "Shiga" antigen the O antigen of *Bact. typhosum* proved to be somewhat refractory to this treatment, and although the protein content of the complex was reduced the hydrolysis could not be induced to go to completion, and the product isolated still contained a nitrogenous component and retained definite antigenic properties. The toxicity of the antigenic complex was not lost during the digestion process. This behaviour is in harmony with the findings of Raistrick and Topley (1934), who were able to isolate antigenic material from *Bact. typhosum* by digestion of the whole acetone-treated organism with trypsin. Freeman and Anderson (1941) have also shown that the tryptic digest-antigen of *Bact. typhosum*, Ty 2, is stable to a prolonged attack by relatively large amounts of trypsin.

Fractionation of the O antigen from formamide solution with alcohol causes a considerable alteration in the polysaccharide-protein ratio of the complex in addition to the primary effect of freeing the complex from phospholipin. This method of treatment would appear to offer a possible method for obtaining the undegraded polysaccharide in a pure state, but fractional precipitations of this kind require much material, and the antigen available has been insufficient for us to make the attempt.

Although the antigenic complex is only sparingly soluble in 85 per cent. or 90 per cent. phenol, the action of this reagent is very similar to that of formamide. The first effect of triturating the complex with strong solutions of phenol is the removal of the phospholipin component. More prolonged extraction presumably leads to a solution of part of the antigen, with subsequent limited dissociation of the complex and gradual removal of at least some of the protein which, in the free state, is readily soluble in this solvent. The whole of the protein moiety, however, could not be removed even after repeated extractions with phenol, and the product, which still contained an appreciable nitrogen content and gave a positive Sakaguchi test for arginine, exhibited definite antigenic activity. Palmer and Gerlough (1940) have made use of the insolubility of the antigen in strong solutions of phenol in their method for isolating the O antigen, but it should be pointed out that agar, and water-soluble agar degradation products, as well as nucleic acid are also insoluble in this solvent, and the presence of these substances is to be expected in the crude phenol-insoluble residue from the direct extraction of agar-grown bacteria.

The O antigenic complex of the typhoid organism behaves like the somatic antigen of *Bact. Shigae* and is partially dissociated in the presence of dilute sodium hydroxide, but unlike the "Shiga" substance, the dissociation of the typhoid complex cannot be readily completed by repeating the process of precipitation with alcohol from alkaline solution. A sample of polysaccharide, however, containing only 0·57 per cent. *N* has been prepared by dissociation. The product proved to be a weak antigen compared with the primary extraction product, and did not give rise to polysaccharide precipitins even after a second course of injections had been given to the group of animals used. The dissociation process can be reversed, and an alkaline solution of the conjugated protein prepared from the somatic antigen of *Bact. Shigae* when mixed with a solution of the undegraded typhoid polysaccharide gave no precipitate on acidification with acetic or trichloracetic acid. The soluble complex formed in this manner proved to be a powerful typhoid O antigen yielding heavily precipitating sera of high agglutinating titre, after a single course of three intravenous injections.

The results of toxicity determinations in mice made with the whole antigenic complexes of *Bact. typhosum* and *Bact. Shigae* and with their components have shown that both primary extraction products are toxic to a roughly equal degree, 0·1–0·2 mg. representing approximately one lethal dose. The O typhoid antigen, unlike that obtained from *Bact. Shigae*, appears to suffer greater loss in toxicity with the removal of the phospholipin component. In one instance the M.L.D. of a phospholipin free O antigen preparation was as high as 0·5 mg. The bulk of the toxicity of the specimens seems to be

associated with the undegraded polysaccharide component, and with one preparation of (*Bact. typhosum*) O antigen the partial removal of the protein component together with some impurities caused an increase in toxicity, the M.L.D. being 0·25 mg. "Shiga" high viscosity polysaccharide is less toxic than the whole antigen, but variation in viscosity in different preparations indicates that it is likely that all the preparations examined were to some extent degraded. The degraded polysaccharide prepared by acetic acid hydrolysis of the antigenic complexes as well as the protein components of both antigens are non-toxic to the extent that comparatively large doses (2 mg.) do not give rise to any noticeable symptoms in mice.

The loss in toxicity on degradation of the "Shiga" high viscosity polysaccharide appears to be bound up with the loss in power to recombine with the protein component and produce an antigen that will again induce the formation of specific immune-body. It seems probable that this relationship also holds for the O polysaccharide component of *Bact. typhosum*. Unfortunately we have been unable to obtain a preparation of undegraded O polysaccharide that is completely free from the protein component and is without antigenic properties. The degraded polysaccharide, on the other hand, is free from N, is not antigenic, and has defied all attempts to render it antigenic by the usual procedures.

In descriptions of the antigens of *Bact. typhosum* and of *Bact. Shigae* prepared by diethyleneglycol extraction we have been careful to point out that the substance in the form in which it was obtained formed heavily opalescent solutions in water. This point was emphasized because it was known that the antigen could exist in other physical states, particularly after the phospholipin had been removed. For example, with the O polysaccharide-protein complex of *Bact. typhosum*, prolonged treatment with organic solvents such as phenol almost certainly changes part of the material to a form which is only soluble with difficulty in formamide and which cannot be dispersed readily in water. This material, like the more readily dispersible forms rapidly dissolved in dilute alkali to give a perfectly clear solution which retain, its activity as an antigen. On acidifying or neutralizing the alkaline solution the opalescence only partially returns, and is then of the bluish type typical of very high dispersions. Heating the solution at 95° C. for 1 hour with 1 per cent. acetic acid causes a flocculent precipitate of conjugated protein to separate in the usual manner. The different appearance in solution of antigenic substances prepared by other methods, such as those which depend on removal of the bacterial proteins by digestion with trypsin (Raistrick and Topley, 1934 ; Freeman, Chalinor and Wilson, 1940), or by precipitation with trichloroacetic acid (Boivin *et al.*, 1933, 1935), must therefore be expected.

Although a direct comparison of our immunologically active fractions with those obtained by other investigators is not possible, it would appear that the acid-insoluble material F 68/P (N, 10·3 per cent.) isolated from *Bact. aertrycke* and described by Stacey, Chalinor and Raistrick (1937) is similar to the conjugated protein substance described in our papers. According to Topley (1937) the fraction F 68/P is very slightly toxic for mice. Injected into rabbits it induces the formation of homologous precipitins, but not of O agglutinins for *Bact. aertrycke*. The former authors state that analogous results were

obtained with antigenic material isolated from the avirulent typhoid bacillus (strain O 901), but no specific chemical or immunological properties have yet been published. It seems probable, however, that these workers were dealing with a substance very similar to the conjugated protein we have isolated from *Bact. Shigae* and *Bact. typhosum* (O 901).

Freeman and Anderson (1941) have recently published a description of the hydrolytic cleavage of an antigenic preparation derived from the strain Ty 2 (O + Vi) of *Bact. typhosum*. These authors were careful to grow their mass cultures on a synthetic medium in order to eliminate sources of contamination from the culture medium. The antigenic substance was isolated after tryptic digestion of the bacterial cell, and although its nitrogen and phosphorus content was significantly higher than that of the substance we have obtained from the strain O 901 by extraction with diethyleneglycol, the hydrolysis products again demonstrate the presence of polysaccharide, lipoid and a water-insoluble nitrogen-containing substance, as components of the antigen. In addition these workers found that a considerable proportion of the nitrogen of the antigenic complex appears in the water-soluble fraction and is soluble in 86 per cent. alcohol. The alcohol-soluble fraction would be expected to contain any low molecular substance liberated by acid hydrolysis, and might possibly contain a fourth component. It is to be noted, however, that the culture of *Bact. typhosum* used by Freeman and Anderson (1941) contained the Vi as well as the O antigen, and although the specific polysaccharide isolated by Freeman and Anderson was shown to be serologically pure O hapten, the components of the Vi complex must, if present in the antigenic material, be liberated during acid hydrolysis, and be contained in one of the fractions isolated. In the absence of any knowledge as to the exact chemical nature of the Vi antigen, or where the components would appear in the fractionation process, the 86 per cent. alcohol-soluble fraction might in part have arisen from the Vi antigen and be not entirely related to the O antigenic complex. Although Freeman and Anderson have isolated an alcohol-soluble nitrogenous component from the antigenic complex of *Bact. typhimurium*, the virulent form of this organism is also known to possess a Vi antigen (Felix and Pitt, 1936; Felix, 1941). In some earlier experiments (Henderson and Morgan, 1938) a considerable amount (3·7 g.) of material was isolated from the "rough" Vi strain, T6S, of *Bact. typhosum* (Felix and Petrie, 1938). Owing to the absence of O and H antigens from this strain and the significant content of Vi antigen, the material isolated is certainly related to the Vi antigenic complex. Indeed, the material induced the formation of Vi agglutinins and in 12 out of 16 rabbits immunized, the antibody titre of the immune sera ranging from 1 : 200 to 1 : 2000. Similarly two horses responded with the production of Vi antibody in titres of 1 : 200 and 1 : 500 respectively. Unfortunately we have had no opportunity of investigating the chemical nature of this antigenic material in detail, but the isolation of a Vi antigenic complex suggests that this same material or its degradation products will be present in preparations of antigenic material made from an O + Vi containing strain such as the one used by Freeman and Anderson.

Although far from complete, the results of the work described in the present paper support the view that the O antigenic complex of *Bact. typhosum*

is similar in type to that of the specific somatic antigen of *Bact. Shigae*, the essential nucleus of which is believed to consist of a polymolecular complex containing polysaccharide and protein molecules. In both instances the native antigen is still more complex and contains a phospholipin moiety. It is not improbable that other components whose nature and function are at present quite unknown are also present in the native complex. It seems unlikely, however, that such components are responsible for any of the dominant immunological properties of the O antigen.

Some preliminary observations indicate that the specific antigen of *Bact. dysenteriae* (Flexner), VI, strain 88 (Boyd, 1940), also consists of a complex of polysaccharide and protein molecules.

SUMMARY.

(1) Antigenic material prepared from *Bact. typhosum* (strain O 901) by extraction with diethyleneglycol has been examined.

(2) The substance possesses the immunological properties of the O antigen of *Bact. typhosum*, and is shown to consist of a polymolecular complex containing three main components, a specific polysaccharide, a protein and a phospholipin.

(3) The protein component is shown to be chemically and immunologically very similar to the conjugated protein prepared from the specific somatic antigen of *Bact. Shigae*.

(4) The toxicity of both antigenic preparations appears to be largely due to the undegraded polysaccharide component, whereas the degraded polysaccharides are practically without toxicity.

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INVESTIGATION INTO THE PRODUCTION OF BACTERIOSTATIC SUBSTANCES BY FUNGI. I. PRELIMINARY EXAMINATION OF 100 FUNGAL SPECIES.

W. H. WILKINS AND G. C. M. HARRIS.

From the Mycology Laboratory, University Department of Botany, Oxford.

Received for publication May 22, 1942.

IN view of the fact that work on the production by fungi of substances which inhibit bacterial growth and development is now being carried on intensively in various parts of the world, it seems desirable to state that such work has been the main subject of investigation in this Laboratory since the early part of 1940. The scope of the investigation is, mainly, the examination of as many fungi as possible with the object of (*a*) determining whether they do or do not produce bacteriostatic substances, (*b*) testing the potency and specificity of the substances produced as indicated by their inhibitory effect on certain representative types of bacteria, and (*c*) examining the conditions which influence the production of the inhibitory substances. This preliminary account deals with the first of these aspects, and, in the interests of economy, is as brief as possible.

The particular objective of the work is the production of bacteriostatic substances of therapeutic significance. The investigation is being done in collaboration with members of the Sir William Dunn School of Pathology at Oxford.

The fungal cultures examined have been obtained, at different times from the Lister Institute, Elstree, Herts, or from the Centraalbureau voor Schimmelcultures, Baarn, Holland. In the list which follows the Lister Number is given in the case of all cultures obtained from there. The fungi are subcultures from an agar stock medium on to liquid culture media in flasks; these are incubated at 20–25° C. and tested at intervals against (1) *Bacterium coli*, (2) *Staphylococcus aureus*, and (3) *Pseudomonas pyocyanea*. The culture media used, the time interval before testing and the method of testing are all subject to alteration and modification; details on this are therefore postponed to a subsequent publication. In the list given below a + sign indicates any degree of inhibition, great or small, that promises to be of significance, and this inhibition may have been produced on any, but not necessarily on all, of the media used. A negative result indicates failure to obtain inhibition of any of the three bacteria at any time on any of the media so far tested.

On any given medium, the production of inhibitory substances varies greatly with different fungi; moreover, with any given fungus the production varies with the medium and, to some extent, with other environmental conditions, particularly temperature. The composition of the medium appears to be of paramount importance, and some of those fungi which, up to the present, have given negative results might reasonably be expected to give positive results if suitable cultural conditions could be established. It seems also that the kind of inhibitory substance produced, as indicated by the type of bacteria inhibited, varies according to the composition of the culture medium on which the fungus has been grown. It is suggested that, within limits, it may be possible to induce a fungus to produce a substance which will effect specific bacterial inhibition, by making a study of its physiological characteristics and modifying the cultural treatment accordingly. This line of investigation is being followed up.

The present list contains fungi of the " Imperfectus " type only, but species from other groups are being similarly examined.

LIST OF FUNGI EXAMINED.

Penicillia.

Positive.	*Bact. coli.*	*Staph. aureus.*	*Pseud. pyocyanea.*
P. chrysogenum Thom. 589	+	+	+
P. citrinum Thom. 6308	..	+	..
P. claviforme Bain. 1718	+	+	+
P. cyclopium Westl. 6310	+	+	..
P. expansum Link. 593	+	+	..
P. funiculosum Thom. 6313	+	+	+
P. spinulosum Thom. 591	..	+	..
P. steckii Zal. 3950	..	+	+
P. vesiculosum Bain. 3567	+	+	..

Negative.

P. brevi-compactum Dierkx. 6306; *P. corymbiferum* Westl. 3609; *P. digitatum* Sacc.; *P. divericatum* Thom. 3089 (= *Paecilomyces varioti* Bain.); *P. frequentans* Westl. 6312; *P. granulatum* Bain. 1720; *P. italicum* Wehm. 6314; *P. lilacinum* Thom. 584; *P. luteum* Zuk. 585; *P. oxalicum* Currie & Thom. 983; *P. pinophilum* Hedg. 1151; *P. puberulum* Bain. 3968; *P. purpurogenum* Fléroff-Stoll. 586; *P. putterillii* Thom. 5621; *P. restrictum* Gillman, 5622; *P. roqueforti* Thom. 588; *P. roseum* Link. 1730 (= *Gliocladium roseum* Bain.); *P. rugulosum* Thom. 592; *P. Schneggii* Boas. 4038; *P. solitum* Westl. 3029; *P. spiculosporum* Lehman. 4042; *P. terrestre* Jensen, 763; *P. varians* Smith. 4152; *P. verrucosum* Dierkx. 3997; *P. wortmanni* Klocker. 5627.

ASPERGILLI.

Positive.	*Bact. coli.*	*Staph. aureus.*	*Pseud. pyocyanea.*
A. cervinus Massee, 5609	+	+	..
A. cinnamomeus Schiemann. 3774	+	+	..
A. effusus Tiraboschi, 973	+	+	..
A. flavipes (Bain. & Sart.) Thom. & Ch. 3890	..	+	..
A. fumaricus Wehm. 1693	+	+	..
A. fumigatus Fres. 367	+	+	+
A. fumigatus mut. helvola Yuill. 5911	+	+	..
A. giganteus Wehm. 3780	+	+	..
A. luchuensis Inui. 1017	+	..	+
A. medius Meiss. 3782	+	+	..
A. nidulans (Eidm.) Winter. 795	..	+	..
A. nidulans var. *alba* Yuill. 5912	..	+	..
A. niger-citricus Wehm. 1692	+	+	..
A. ochraceus Wilh. 979	+	+	..
A. parasiticus Speare. 975	+	+	..
A. schiemannii (Schiemann) Thom. 2044	+	+	+
A. tamarii Kita. 599	..	+	..
A. terreus Thom. 981	+	+	..

Negative.

A. amstelodami (Mang.) Thom. & Ch. 1945 ; *A. candidus* Link. 595 ; *A. carbonarius* (Bain.) Thom. 1325 ; *A. clavatus* Desm. 978 ; *A. conicus* Blochwitz. 3775 ; *A. disjunctus* Bain. & Sart. 3776 ; *A. ferrugineus* Fuckel. 3777 ; *A. ficuum* (Reich.) Henn. 3308 ; *A. fischeri* Wehm. 3779 ; *A. flavus* Link. 596 ; *A. glaucus* (group) Link. 2655 ; *A. minimus* Wehm. 3783 ; *A. mollis* Bain. & Sart. 3784 ; *A. nidulans* var. *nicollei* Pinoy, 3786 ; *A. niger* v. *Tiegh.* 594 ; *A. okazakii* Okazaki, 3787 ; *A. oryzae* (Ahlb.) Cohn. 598 ; *A. ostianus* Wehm. 3788 ; *A. pulverulentus* (McAlp.) Thom. 1324 ; *A. repens* (Cda.) Sacc. 794 ; *A. restrictus* Smith. 4155 ; *A. restrictus* var. *B.* Smith, 4156 ; *A. scheelei* Bain. & Sart. 3790 ; *A. sydowi* (Bain. & Sart.) Thom. & Ch. 980 ; *A. terricola* var. *americana* Marchal. 974 ; *A. ustus* (Bain.) Thom. & Ch. 3757 ; *A. versicolor* (Vuill.) Tiraboschi. 1883 ; *A. violaceofuscus* Gasperini, 5792 *A. wentii* Wehm. 597.

FUNGI IMPERFECTI.

Positive.	*Bact. coli.*	*Staph. aureus.*	*Pseud. pyocyanea.*
Botrytis cinerea Pers.	..	+	..
Fusarium javanicum Kds. 2862	+	..	..
Helminthosporium avenae Eidam.	..	+	..

Negative.

Alternaria Citri Ellis & Pierce, 2863 ; *Arthrobotrys oligospora* Fres ; *Ascochyta pisi* Lib. ; *Cladosporium herbarum* Link. ; *Colletotrichum lini* (Westerdijk.) Tochinai ; *Epicoccum oryzae* Ito et Iwadare ; *Fusarium coeruleum* (Lib.) Sacc. ; *F. culmorum* (W. Sm.) Sacc. ; *Gleosporium fructigenum* Berk. 2513 ; *Oidium aurantium* ; *Pestalozzia macrotricha* ; *Spegazzinia ornata* Sacc. ; *Sphaeropsis malorum* Peck ; *Stysanus stemonitis* (Pers.) Cda. 1129 ; *Trichoderma lignorum* (Tode) Harz. ; *Tricothecium roseum* Link.

From the above it seems that the Aspergilli are the most promising as they give approximately 40 per cent. positive results. The Penicillia show positive results in about 25 per cent. of those tried, while the Fungi Imperfecti are mostly negative.

In that this paper involves no discussion, reference to previous literature is limited to the paper "Antagonistic Relations of Micro-organisms," by Waksman in *Bacteriological Reviews*, 1941, **5**, 231, which contains a comprehensive and up-to-date bibliography on relevant subject-matter.

The Senior Author is indebted to the Nuffield Research Fund for a grant enabling the Junior Author to be employed as Research Assistant, and to the Air Ministry for releasing him for that purpose. Both Authors are grateful for the co-operation of Prof. Florey and his co-workers at the School of Pathology and to the Director of the Lister Institute and his Staff. They are also very appreciative of the help received from Mr. A. H. Cripps, the Technical Assistant in this Laboratory.

THE ANTIGENICITY OF NON-PRECIPITATING COMPLEXES.

F. C. BAWDEN AND A. KLECZKOWSKI.

From the Rothamsted Experimental Station, Harpenden, Herts.

Received for publication May 23, 1942.

WHEN some protein antigens are heated in the presence of other, serologically unspecific, protein they cease to be precipitated by their antisera, although they still combine with antibodies for they inhibit the precipitation of unheated antigens. This change in behaviour is apparently brought about by the combination of the antigen and other protein during the early stage of heat denaturation to form a complex, which after combination with antibody still remains soluble (Bawden and Kleczkowski, 1941*a* and *b*). In this paper the antigenicity of such complexes *in vivo* and their behaviour in complement-fixation tests are described.

MATERIALS AND METHODS.

Tomato bushy stunt virus and human serum whole globulin were used as antigens and rabbit serum albumin as the unspecific protein. These were all prepared by methods previously described. Virus-albumin complex was prepared by heating 0·1 per cent. virus solution in the presence of 0·5 per cent. rabbit albumin for 10 minutes at 83° or 95° C. Globulin-albumin complex was prepared by heating 0·1 per cent. solution of human globulin in the presence of 0·5 per cent. rabbit albumin for 10 minutes at 83° C., or in the presence of 0·2 per cent. rabbit albumin for 5 minutes at 100° C. All the solutions were heated in 0·9 per cent. NaCl at pH 7·0.

Antisera were prepared by injecting rabbits intravenously 6–10 times every third day with 2 ml. of the following solutions :

No. 1. 0·1 per cent. solution of unheated bushy stunt virus.

No. 2. Virus-albumin complex prepared by heating at 83° C.

No. 3. Virus-albumin complex prepared by heating at 95° C.

No. 4. 0·1 per cent. solution of bushy stunt virus heated for 10 minutes at 95° C.

No. 5. 0·5 per cent. solution of unheated human serum whole globulin.

No. 6. Globulin-albumin complex prepared by heating at 83° C.

No. 7. Globulin-albumin complex prepared by heating at 100° C.

Precipitation and inhibition tests and tests of infectivity were all made by the methods previously described. In the complement-fixation tests serial dilutions of antigens were made in ordinary test tubes, 0·5 ml. of antigen solutions being used. 0·5 ml. of guinea-pig serum (complement) diluted 1/10 was added, and this was followed by 0·5 ml. of antiserum appropriately diluted, or by 0·5 ml. of saline in the antigen control series. The tubes were shaken and left at room temperature for one hour. 0·5 ml. of 5 per cent. suspension of washed sheep cells and 0·5 ml. of suitably diluted amboceptor were then added, after which the tubes were shaken and transferred to a room at 37° C. The readings were made as soon as there was complete haemolysis in the control series.

RESULTS.

Antisera to Non-precipitating antigen complexes.

Bushy stunt virus.

Table I shows the results of precipitation tests made with antisera prepared by immunizing rabbits with unheated bushy stunt virus (No. 1) and with the virus-albumin complex formed at 83° C. (No. 2). The test antigens were those used for immunization and 0·1 per cent. bushy stunt virus solution heated for 10 minutes at 83° C.

It will be seen that the virus-albumin complex was active as a producer of precipitating antibodies *in vivo*. These antibodies behaved in the same way as those produced by injecting the virus alone and failed to precipitate the complex used to produce them. No differences were detected between the behaviour of the two antisera. Both precipitated with the virus, giving a higher titre with heated than with unheated virus, and the antibodies of both antisera combined with the virus-albumin complex, the combination inhibiting the precipitation of virus subsequently added.

There was also no significant difference between the ability of the two antisera to neutralize the infectivity of the virus *in vitro* (Table II). 1 ml. of 0·002 per cent. virus solution was mixed with 1 ml. of the antisera diluted 1/10 and 1/50, and tested for their infectivity on *Nicotiana glutinosa* after 1 hour. The serum prepared by injecting the non-infective and non-precipitating virus-complex (No. 2) reduced the infectivity much more than a normal rabbit serum, and only slightly less than that prepared against fully active virus (No. 1).

The results given in Tables I and II show that heating for 10 minutes at 83° C. has little or no effect on the antigenicity of bushy stunt virus. We have

TABLE I.—*Precipitation and Inhibition with Antisera to Bushy Stunt Virus and to Virus-Albumin Complex Prepared by Heating at 83° C.*

In the precipitation tests 1 ml. of antigen at constant virus content (0·005 per cent.) was added to a series of tubes each containing 1 ml. of antiserum at different dilutions. + indicates precipitation. − indicates no precipitation.

After 3 hours' incubation the tubes containing the virus-albumin complex were tested for inhibition. 0·1 ml. of (0·05 per cent.) virus solution was added to the tubes in which there was no precipitation. i indicates that there was inhibition (no precipitate separated); o indicates that there was no inhibition (a precipitate separated). The same results were got, whether unheated virus or virus heated at 83° C. was added to test for inhibition.

Antigen.	Antiserum No. 1 to unheated virus. Serum dilution.								Anti-serum No. 2 to virus-albumin complex prepared by heating at 83° C. Serum dilution.						
	1/10.	1/20.	1/40.	1/80.	1/160.	1/320	1/640.	Saline control.	1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
Unheated virus	+	+	+	+	+	+	−	..	+	+	+	+	+	+	−
Heated virus	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+
Virus-albumin complex	−	−	−	−	−	−	−	..	−	−	−	−	−	−	−
	o	i	i	i	i	i	i	..	o	i	i	i	i	i	i

TABLE II.—*Neutralization of Infectivity with the Antiserum to Unheated Bushy Stunt Virus (No. 1) and to the Virus-Albumin Complex (No. 2).*

1 ml. of 0·002 per cent. virus solution mixed with 1 ml. of—			Average number of lesions per leaf.
Saline			277
Normal serum	Diluted	1/10	151
Anti-serum No. 1	,,	1/10	1
,, No. 2	,,	1/10	3
Normal serum	,,	1/50	175
Anti-serum No. 1	,,	1/50	6
,, No. 2	,,	1/50	13

shown previously that that amount of heating also has little effect on its ability to combine with antibodies *in vitro*. After heating for 10 minutes at 95° C., however, we could obtain no evidence that the virus combined with antibodies *in vitro*. After heating with rabbit serum albumin at this temperature the stable suspension did not inhibit the precipitation of unheated virus by its antiserum, and after heating alone, the denatured, insoluble material did not absorb measurable amount of antibodies when incubated with virus antiserum (Bawden and Kleczkowski, 1941*a*). Nevertheless, it seems that heating sufficient to destroy serological reactions *in vitro* does not destroy all antigenicity. We have injected rabbits with suspensions of virus heated alone for 10 minutes at 95° C. and with virus-albumin complex formed by heating at 95° C. The results of precipitation and inhibition tests with the antisera obtained in this way are shown in Table III. It will be seen that the two

TABLE III.—*Precipitation and Inhibition with Antisera Prepared against Bushy Stunt Virus, Heated alone and with Albumin at 95° C.*

The method of test as described in Table I.
Inhibition was tested by adding virus solution heated at 83° C.

Antigen.	Anti-serum No. 4 to virus heated alone. Serum dilution. 1/10.	1/20.	1/40.	1/80.	Anti-serum No. 3 to virus heated with albumin. Serum dilution. 1/10.	1/20.	1/40.	1/80.
Unheated virus	+	−	−	−	+	−	−	−
Virus heated at 80° C.	+	+	−	−	+	+	−	−
,, ,, 83° C.	+	+	+	−	+	+	+	−
Virus-albumin complex formed at 83° C.	−	−	−	−	−	−	−	−
	i	i	i	..	i	i	i	..
Virus-albumin complex formed at 95° C.	−	−	−	−	−	−	−	−
	o	o	o	..	o	o	o	..

antisera behaved similarly; they precipitated the virus heated alone at 80° or 83° C. and the unheated virus, and their antibodies combined with virus-albumin complex formed by heating at 83° C., but there is no evidence that they reacted with the material used for immunizing. In other words, these sera reacted qualitatively like those prepared against unheated virus. A quantitative difference was indicated by the lower precipitation titre, and this has been a constant feature of sera prepared against such heated viruses. Several rabbits have been injected with different virus preparations heated for 10 minutes at 95° C. All these heated preparations have been antigenic but produced antisera with much lower titres than those prepared against unheated virus. As there is no evidence of any change in antigenic specificity after heating, it seems reasonable to assume that the effect is due to antigens present in the unheated virus that survive the heating in quantities too small to be detected *in vitro* but sufficient to stimulate antibody production *in vivo*.

Tobacco mosaic virus behaves somewhat similarly when heated for 10 minutes at 95° C. In the presence of rabbit albumin a stable suspension is produced, whereas when heated alone insoluble material separates. Rabbits injected with such heated virus produce antibodies that precipitate unheated virus, and these sera, like those produced against heated bushy stunt virus, have much lower precipitation titres than those produced against unheated virus. Antisera produced in this way contain no antibodies reacting specifically with heated virus, and the antigenicity of the heated virus seems due to original antigenic groups that survive the heating. Such antisera are readily absorbed completely by unheated virus, whereas large quantities of the heated virus are necessary for this. Thus heating produces no new serological specificity.

Human serum globulin.

Table IV shows the results of precipitation tests with antisera Nos. 5 and 6, prepared respectively against unheated human globulin and a non-precipitating globulin-albumin complex formed by heating a mixture of human globulin and rabbit albumin for 10 minutes at 83° C. The results are exactly similar to

TABLE IV.—*Precipitation and Inhibition with Antisera to Human Serum Globulin and to Globulin-Albumin Complex Prepared by Heating at 83° C.*

1 ml. of each antiserum diluted 1/20 was added to 1 ml. of varying dilutions of antigen.

Inhibition was tested after 3 hours' incubation by adding 0·1 ml. of 0·05 per cent. of unheated globulin solution.

The symbols as used in Table I.

Antigen.	Anti-serum No. 5 to unheated globulin. Amounts of human globulin (in mg.).							Anti-serum No. 6 to globulin-albumin complex. Amounts of human globulin (in mg.).						
	0·5.	0·25.	0·12.	0·06.	0·03.	0·015.	0·0075.	0·5.	0·25.	0·12.	0·06.	0·03.	0·015.	0·0075.
Unheated globulin	+	+	+	+	+	+	−	−	−	−	+	+	+	+
Globulin-albumin complex	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	i	i	i	i	o	o	o	i	i	i	i	i	i	o
Heated globulin	+	+	+	+	−	−	−	−	+	+	+	+	+	+

TABLE V.—*Precipitation and Inhibition with Antiserum No. 7 to Globulin-Albumin Complex Prepared by Heating at 100° C.*

1 ml. of the antiserum diluted 1/20 was added to 1 ml. of varying dilutions of antigen.

Inhibition was tested after 4 hours' incubation by adding 0·25 ml. of 0·05 per cent. human globulin solution heated at 100° C.

The symbols as used in Table I.

Antigen.	Amounts of human globulin (in mg.).						
	0·5.	0·25.	0·12.	0·06.	0·03.	0·015.	0·0075.
Unheated globulin	−	−	−	−	−	−	−
	i	i	i	i	i	o	o
Globulin-albumin complex	−	−	−	−	−	−	−
	i	i	i	i	i	o	o
Heated globulin	−	+	+	+	+	+	−
Unheated rabbit albumin	−	−	−	−	−	−	−
	o	o	o	o	o	o	o
Heated rabbit albumin	−	−	−	−	−	−	−
	o	o	o	o	o	o	o

those obtained with bushy stunt virus, the serum produced against the complex being unable to precipitate the material used for immunization and reacting qualitatively like the antiserum against normal globulin. Unheated globulin and globulin heated alone at 83° C. were precipitated by both antisera, but the globulin-albumin complex was precipitated by neither.

A serum produced against a globulin-albumin complex prepared by heating for 5 minutes at 100° C. (No. 7), however, gave rather different results ; these are shown in Table V. This serum again did not precipitate with the material used for immunization, but it did precipitate the globulin heated alone for 5 minutes at 100° C., although it did not precipitate the unheated globulin. However, the unheated globulin combined with the antibodies produced by the complex for it inhibited the precipitation of globulin heated alone at

100° C. Thus the globulin-albumin complex heated at 100° C. contained antigenic groups of normal globulin, but the antibodies produced by them did not precipitate the normal globulin. The significance of this will be discussed later.

None of the sera produced by injecting complexes containing rabbit albumin gave any reaction, either precipitation or inhibition, with heated or unheated rabbit albumin or globulin. One test with rabbit albumin is shown in Table V.

Complement fixation by non-precipitating antigens.

Complement-fixation tests show no differences between the antiserum against unheated bushy stunt virus (No. 1) and that against a virus-albumin complex formed by heating for 10 minutes at 83° C. (No. 2). Table VI shows the results with the serum No. 2 ; precisely similar results were obtained with the serum No. 1. It will be seen that the non-precipitating complex fixed complement with both sera to the same extent as unheated virus, and that fixation by the virus heated alone for 10 minutes at 83° C. was slightly less.

Table VII shows the results of similar complement-fixation tests with the antiserum to unheated human whole globulin (No. 5). Although the globulin heated alone for 10 minutes at 83° C. was precipitated by the antiserum, whereas the globulin-albumin complex formed by heating for 10 minutes at 83° C. was not, both fixed complement equally and only slightly less than the unheated globulin.

The fixation of complement equally by the same antisera with precipitating and non-precipitating antigens shows clearly that fixation is independent of the separation of insoluble material. Our results with heated antisera, however, suggest that something further than mere union between antigen and antibody may be necessary for fixation to occur (Bawden and Kleczkowski, 1942).

Complement fixation is obviously the most suitable test for demonstrating the antigenicity of these non-precipitating complexes. The antigenicity of those we have studied can also be demonstrated by using the precipitable components of the complexes as test antigens in precipitation tests. Complexes with serological behaviour similar to those we have prepared by heating protein mixtures may also occur in nature, when the precipitable component might not be available alone. For example, many plant proteins appear to be relatively inert antigenically, and it is possible that combination between these or other inert substances and viruses would give non-precipitable complexes. Chester (1937), working with sap from plants infected with different viruses and using the precipitation test, got no evidence that many were antigenic. No doubt some failed because the concentration of virus was insufficient to give a visible precipitate, but the possibility that others were present in non-precipitating forms is also worth consideration. Whatever the explanation, the application of complement fixation in virus work is likely to extend the number of viruses amenable to serological examination, for in addition to revealing non-precipitating antigens, the method is more sensitive than the precipitation test. In our tests we were able to get positive complement fixation with about one-tenth of the virus concentration necessary to give precipitation reactions.

TABLE VI.—*Complement Fixation with Antiserum No. 2 to Bushy Stunt Virus-Rabbit Albumin Complex Formed by Heating for 10 minutes at 83° C.*

++++ indicates complete fixation (no haemolysis); − indicates no fixation (complete haemolysis).
+++, ++ and + indicate intermediate degrees of fixation.

Antigen.	Dilution of anti-serum.	Concentrations of antigen in 10^{-4} g. of virus protein per 1 ml.					
		1·0.	0·3.	0·1.	0·03.	0·01.	0.
Unheated virus	1/15	++++	++++	+++	++	+	−
	1/45	++++	++++	++	++	+	−
	1/135	++	+++	++	+	−	−
	Saline	−	−	−	−	−	−
Heated virus	1/15	++++	+++	+	−	−	−
	1/45	++++	++	+	−	−	−
	1/135	++	+++	+	−	−	−
	Saline	−	−	−	−	−	−
Virus albumin complex	1/15	++++	++++	++++	++	+	−
	1/45	++++	++++	+++	++	+	−
	1/135	++	+++	++	+	−	−
	Saline	−	−	−	−	−	−

TABLE VII.—*Complement Fixation with Antiserum No. 5 to Unheated Human Globulin.*

Symbols as in Table VI.

Antigen.	Dilution of anti-serum.	Concentration of antigen in 10^{-4} g. of human globulin per 1 ml.					
		1·0.	0·5.	0·25.	0·12.	0·06.	0.
Unheated globulin	1/20	++++	++++	++++	+++	+	−
	1/60	++	+++	++	+	−	−
	Saline	−	−	−	−	−	−
Globulin-albumin complex	1/20	++++	+++	++	+	−	−
	1/60	+++	++	−	−	−	−
	Saline	−	−	−	−	−	−
Heated globulin	1/20	++++	++++	+++	++	−	−
	1/60	+++	++	−	−	−	−
	Saline	−	−	−	−	−	−

DISCUSSION.

Although the precipitin test is the most used serological reaction, the production of a precipitate is by no means a necessary sequel to the combination of antibody and antigen. Whether or not precipitation occurs will depend on the solubility of the product of combination, which in turn will depend on the extent to which combination has covered the hydrophilic groupings on antigen and antibody particles. This is so even with normal precipitating antigens, for they precipitate only over a limited range of antigen/antibody ratios.

As a result of our earlier tests with the heat-produced non-precipitating complexes, we compared their behaviour to that of haptenes. Whether haptenes fix complement does not seem to have been studied, but as our complexes stimulate the production of antibodies *in vivo* they clearly differ from haptenes and warrant the name antigen.

When a non-precipitating haptene is coupled with a serologically unspecific protein, the complex generally becomes a precipitating antigen. At first sight this seems contrary to our results, showing that precipitating antigens are changed into non-precipitating antigens when coupled with other protein, but so little is known about the nature and mechanism of serological reactions that there may be no real contradiction. The tendency of a protein to stay in solution under any given set of conditions must depend on the proportion of its total surface that is covered with hydrophilic groups. Any agent that covers sufficient of these groups would cause precipitation, and it is probable that in the normal antigen-antibody reactions there is considerable mutual covering of such groups. In the systems we have described in this paper antigens are combined with other proteins, and this could reasonably be expected to increase the number of hydrophilic groups in the complex, and to introduce some that would remain unaffected as a result of combination with antibody. Thus it becomes less likely that such combination would lead to the formation of insoluble particles. When haptenes are coupled with proteins, their changed behaviour in serological tests shows that a greater covering of hydrophilic groups now occurs on combination with antibody. Whether this is simply a result of the large complex particle covering more of the antibody surface than did the simple haptene or to some more fundamental change cannot be indicated.

In our tests we have no results that necessitate postulating new antigenicity as a result of heating. Heating around 80° C. either alone or with albumin to form complexes had no demonstrable effect on the antigenicity of our antigens. Heating sufficient to denature completely greatly reduced the antigenicity of both tomato bushy stunt and tobacco mosaic viruses, so that only very weak sera could be prepared by injecting them into rabbits. These sera contained no antibodies incapable of combining with the unheated viruses, and the formation of antibodies against the viruses heated for 10 minutes at 95° C. seems solely a result of some antigenic groups surviving the heating.

Furth (1925), working with serum proteins, claimed that heating for 5 minutes at 100° C. destroyed the original specificity and produced a new heat specificity. We have confirmed some of his experimental results, but consider these are better interpreted by the formation of non-precipitating complexes during heating and by some antigenicity surviving heating than by a new heat-induced specificity. Furth drew his conclusions from the facts that antisera to heated sera precipitate heated but not unheated homologous sera, that heated sera do not precipitate with antisera to unheated homologous sera, and from the existence of some degree of group specificity of heated unrelated proteins.

We found that human globulin, converted into a non-precipitating complex by heating for 5 minutes at 100° C. in the presence of rabbit albumin, produced an antiserum different from those produced by unheated human globulin, for it precipitated with heated but not with unheated human globulin. But the unheated globulin did combine with these antibodies, for it inhibited the precipitation of heated globulin, and this seems incompatible with the view that antibodies produced by heat-changed serum protein are unspecific for the unheated protein. The failure of unheated globulin to be precipitated by

the antiserum produced against the globulin-albumin complex formed by heating at 100° C. is more likely due to the destruction of certain antigenic groups of the globulin. If this is so, the antiserum would contain antibodies only for some of the more heat-resistant groups, and only a reduced part of the surface of unheated globulin particles would be involved in the antigen-antibody reaction, so that the solubility of the product of combination would be too great for precipitation to occur. The much less soluble, heated globulin, with a reduced surface volume ratio, would be expected to precipitate by combination with these antibodies.

The failure of heated serum proteins to precipitate with antisera to unheated proteins can be attributed to the formation of non-precipitating complexes, without assuming complete destruction of the original specificity. Solutions of human globulin and albumin heated separately for 5 minutes at 100° C. precipitate with antisera to unheated globulin and albumin respectively (Bawden and Kleczkowski, 1941*a*). When the proteins are heated in the presence of an unspecific protein (rabbit albumin), however, complexes are formed, and these do not precipitate but give the inhibition phenomenon, which was also noticed by earlier workers (Spiegel-Adolf, 1926). Heating human globulin in the presence of human albumin also leads to the formation of a complex, which does not precipitate but which gives the inhibition phenomenon with antisera containing antibodies only to human globulin. It is precipitated by the antisera containing antibodies to human albumin. Immunization with a whole serum often leads to production of antibodies predominantly or even exclusively against the globulin fraction (Kleczkowski, 1938), and for such antisera the albumin fraction of the antigenic serum represents a non-specific protein liable to form a non-precipitating complex with the globulin.

It is also possible that antiserum to unheated protein may contain predominantly or exclusively antibodies to heat-labile antigenic groups. Such antisera cannot give any serological reaction with the heated protein, but even so there is no need to postulate any " new " heat-induced antigenic groups.

Furth claims to have demonstrated group specificity for heated unrelated proteins, for he states that heated rabbit serum gives a weak precipitation with antisera produced by injecting rabbits with heated sera from other species. This we were unable to confirm. Antiserum to human globulin-rabbit albumin complex formed by heating at 100° C. (No. 7) gave no reaction (neither precipitation nor inhibition) with either heated or unheated rabbit serum albumin and globulin.

SUMMARY.

Bushy stunt virus and human serum globulin remain antigenic when changed into non-precipitating complexes by heating with rabbit albumin in suitable conditions. The injection of such complexes into rabbits produces antisera apparently identical in all their serological reactions with those produced against the normal virus or globulin. Solutions of the non-precipitating antigens fix complement with antisera as strongly as solutions of the normal precipitating antigens, showing that complement fixation does not depend on the formation of insoluble material. Sufficient heating destroys

most of the original antigenicity of bushy stunt virus and globulin, but there is no evidence that heating creates any new specificity.

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THE EFFECTS OF HEAT ON THE SEROLOGICAL REACTIONS OF ANTISERA.

F. C. BAWDEN AND A. KLECZKOWSKI.

From the Rothamsted Experimental Station, Harpenden, Herts.

Received for publication May 23, 1942.

DURING the early stages of heat denaturation, precipitating antibodies can combine and form complex particles with other proteins undergoing denaturation simultaneously. The serological behaviour of the complex particles in precipitation tests depends on the type and quantity of proteins with which the antibody has formed a complex. Euglobulin complexes, formed when the euglobulin fractions of antisera are heated alone, behave very much like unchanged antibodies. On the other hand, mixed complexes, formed by heating antibodies with albumin, no longer precipitate their antigens, although they still combine with them. Antisera partly denatured by heat behave like mixtures of antibody particles with different properties, all able to combine with antigen, but only some able to cause precipitation. There is competition between the different antibody particles for the available antigen, and the mixed complexes inhibit the precipitation by the remaining precipitating antibodies. The degree of this inhibition varies with different antigens. It is considerable with those giving O-type granular precipitates, but it is very slight with those giving H-type nebulous floccules (Kleczkowski, 1941).

In this paper the abilities of heated antisera and antiserum fractions to fix complement and neutralize infectivity are described and compared with their ability to cause precipitation. Tobacco mosaic virus was used as an H-type antigen, and tomato bushy stunt virus and human serum globulin as O-type antigens. The antigens, antisera and complexes were prepared and tested by methods previously described (Kleczkowski, 1941 ; Bawden and Kleczkowski, 1942).

RESULTS.

Precipitation and complement fixation.

The results of precipitation and complement-fixation tests using bushy stunt and tobacco mosaic viruses with their antisera heated at various temperatures are given in Tables I and II. The sera were diluted 1/10 in saline before heating and were heated for 10 minutes. Heating in other conditions gives different results, for the formation of complexes is greatly influenced by the concentration of proteins. There are no differences in the behaviour of the two heated antisera in complement-fixation tests, whereas in the precipitation tests there are considerable differences. The differences are most obvious after heating at 75° C., when both sera still fix complement strongly and tobacco mosaic virus antiserum precipitates well, whereas bushy stunt virus antiserum no longer precipitates but inhibits strongly. Two main factors seem responsible for these similarities and differences in the serological reactions of the heated antisera to the different type antigens: (1) Mixed complexes neither fix complement nor interfere with the fixation of complement by other forms of antibodies, although they can still combine with antigen. (2) Mixed complexes greatly inhibit the precipitation of bushy stunt virus by precipitating antibodies, but not the precipitation of tobacco mosaic virus.

The results with bushy stunt virus antiserum heated at 80° C. suggest that mixed complexes do not fix complement, for this serum has a high inhibition titre, showing a large content of non-precipitating complexes, but fixes complement only weakly, no more than might be expected from antibodies not changed into mixed complexes. That the presence of mixed complexes does not interfere with the ability of other antibody particles to fix complement with their antigen is shown by the results with mixtures of heated and unheated antisera (Table II). Mixtures containing equal parts of heated and unheated sera fix complement just as effectively as the unheated fraction alone. This is very different from the results of precipitation tests with such mixtures of sera. With bushy stunt virus only a small proportion of mixed complexes is needed to inhibit precipitation, and the precipitation titres of mixtures of heated and unheated sera are much less than would be expected from the content of unchanged antibodies. This is not so with tobacco mosaic virus, where at least four times as much heated as unheated serum is needed in a mixture before any reduction in the precipitation titre of the unheated serum is obtained (Kleczkowski, 1941).

Thus the results in Tables I and II can be interpreted in the following manner. Heating at 75° C. produces some mixed antibody complexes in both sera, and this is reflected in the slight drop in complement-fixation power; the proportion of mixed complexes is sufficient to inhibit the precipitation of bushy stunt virus but not that of tobacco mosaic virus. Heating at 80° C. changes most of the antibodies into mixed complexes, and the ability to fix complement is almost destroyed; the proportion of non-precipitating antibodies is also sufficient to prevent the precipitation of even tobacco mosaic virus. Heating at 90° C. destroys all the ability of the complex to combine with antigen.

TABLE I.—*The Effect of Heat on the Precipitation Power of Antisera.*

The antisera were heated for 10 minutes at a dilution 1/10 in physiological saline.

The antigen solutions were used at 0·0025 per cent. for tobacco mosaic and 0·005 per cent. for bushy stunt virus. 1 ml. of antigen solution was added to a series of tubes each containing 1 ml. of antiserum at varying dilutions. + indicates precipitation and − no precipitation.

After 3 hours at 50° C., 0·1 ml. of control antiserum at a dilution 1/8 was added to the tubes in which there was no precipitation. i indicates that there was still no precipitation (inhibition), and o that a precipitate was formed (no inhibition).

Temperature of heating.	Dilution of the antisera. Tobacco mosaic virus. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.	1/1280.	Bushy stunt virus. 1/10.	1/20.	1/40.	1/80.	1/160.	1/320.	1/640.
Unheated	+	+	+	+	+	+	+	− o	+	+	+	+	+	+	− o
70° C.	+	+	+	+	+	+	+	− o	+	+	+	+	+	− o	− o
75° C.	+	+	+	+	+	+	− o	− o	− i	− i	− i	− i	− o	− o	− o
80° C.	− i	− o	− o	− o	− o	− o	− o	− o	− i	− i	− i	− i	− i	− o	− o
90° C.	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o	− o

Although antisera to O-type antigens heated for 10 minutes between 75° and 80° C. fail to cause precipitation, it was shown that this did not necessarily imply the absence of precipitating antibodies, for the failure to cause precipitation could result from the strong inhibiting action of mixed complexes (Kleczkowski, 1941). We have now demonstrated the presence of precipitating antibodies in such heated sera by isolating fractions able to cause precipitation. Table III shows the results of such experiments with bushy stunt virus antiserum diluted 1/10 in saline and heated for 10 minutes at 75° and 80° C. After heating, the serum ceased to precipitate and inhibited precipitation. The serum heated at 75° C. was fractionated by salting-out with ammonium sulphate, and the successive fractions obtained at 33, 50 and 100 per cent. saturation were redissolved in saline in the volume from which they were separated, and tested for their ability to precipitate and to inhibit the precipitation of the virus. It will be seen that the protein separated at 33 per cent. saturation gave strong inhibition, that at 50 per cent. gave precipitation and that at 100 per cent. neither precipitation nor inhibition. Thus from a serum that as a whole failed to precipitate, a fraction was isolated with globulin-like properties and able to cause precipitation, showing that heating had not changed all antibodies into non-precipitating complexes.

TABLE II.—*The Effect of Heat on the Complement-Fixation Power of Antisera.*

The antisera were heated for 10 minutes at a dilution 1/10 in physiological saline.
++++ indicates complete fixation (no haemolysis), − indicates no fixation (complete haemolysis), and +++, ++ and + indicate intermediate degrees of fixation.

Temperature of heating of antiserum.	Dilution of antiserum.	Concentration of antigen in 10^{-4} g. per ml.											
		Tobacco mosaic virus.						Bushy stunt virus.					
		1·0.	0·3.	0·1.	0·03.	0·01.	0.	1·0.	0·3.	0·1.	0·03.	0·01.	0.
Unheated	1/20	..	..	..	..	..	..	++++	++++	++++	+++	+++	−
	1/40	+++	++++	++++	++++	+++	−	++++	++++	++++	+++	+++	−
	1/80	++	+++	++++	++++	+++	−	..	..	..	..	..	..
75° C.	1/20	+++	++++	++++	++++	++++	−	++++	++++	++++	++++	+++	−
	1/40	+	+++	++++	++++	+++	−	+	+++	++++	++++	+++	−
80° C.	1/20	−	+	++	+++	+++	−	−	+	+++	+++	++	−
	1/40	−	−	−	+	++	−	−	−	+	++	+	−
90° C.	1/20	−	−	−	−	−	−	−	−	−	−	−	−
80° C. Unheated (mixed)	1/20 } 1/20 }	+++	++++	++++	++++	+++	−	++++	++++	++++	++++	+++	−
	Saline	−	−	−	−	−	−	−	−	−	−	−	−

Similarly, small amounts of precipitating antibodies were recovered from antiserum heated at 80° C., by centrifuging for 15 minutes at 15,000 r.p.m. The yellow gelatinous sediment redissolved to give a solution that precipitated the virus, while the supernatant fluid still inhibited strongly. The precipitating antibodies separated in this way were heat changed, for they sedimented when centrifuged at 15,000 r.p.m. ; they behaved much like " euglobulin complexes " previously described (Kleczkowski, 1941), giving a specific precipitate of more fluffy appearance than that caused by unchanged antibodies.

Heating bushy stunt virus antisera has given more constant results than heating tobacco mosaic virus antisera, probably because of the greater sensitivity of the bushy stunt virus-antibody system to the presence of mixed antibody complexes, and the results in Table I are typical of all bushy stunt virus antisera. Heating for 10 minutes at 90° C. always destroys all serological reactions for both antisera. Some antisera to tobacco mosaic virus, however, after heating for 10 minutes at 85° C. still precipitate the antigen; some precipitate after heating at 80° C., while others, like that given in Table I, fail to precipitate after heating at 80° C. Similarly, no inhibition occurs with some sera, whereas it does with others, although always very much less than with antisera to O-type antigens. These variations seem to be due to differences in the ratio of precipitating antibodies and globulin to other proteins in the sera. Only when the precipitation titre of the antiserum is low is precipitability lost by heating at 80° C. and is the effect of mixed complexes sufficient to give inhibition.

Tables IV and V give the results of precipitation and complement fixation experiments with the euglobulin fraction of the two virus antisera after being heated alone and in the presence of excess albumin. The euglobulin, containing antibodies, was used at 0·1 per cent., the albumin at 0·5 per cent. and the solutions were heated for 10 minutes at 75° C. The euglobulin fractions and the mixtures were heated to the same amount and so presumably had the same amount of antibody denatured, yet the serological reactions suffered much more in the mixtures than in the euglobulin heated alone. Heating euglobulin fractions of the antisera alone has little or no effect on the ability of antibodies to cause precipitation, although they form " euglobulin complexes " with zones of precipitation and the character of the precipitate different from those given by unchanged antibodies (Kleczkowski, 1941). Table V shows that if euglobulin fractions of the antisera are heated alone, there is little change in their ability to fix complement, so that it seems that the " euglobulin complexes " both fix complement with and precipitate their antigens. The changes in ability to cause precipitation after heating with albumin have already been shown to result from the precipitating antibodies forming non-precipitating complexes with the albumin. The results in Table V suggest that antibodies responsible for complement fixation also react with albumin to form complexes unable to fix complement. The fact that the heated mixtures still fix complement to some extent can be attributed to the presence of residual unchanged antibodies or some euglobulin complexes.

Experiments with antisera to human globulin gave results essentially similar to those with bushy stunt virus antisera. They provide more clear-cut evidence, however, that non-precipitating antibody complexes cannot fix

TABLE III.—*Separation of Precipitating Fractions from Heated Bushy Stunt Virus Antisera.*

1 ml. of 0·005 per cent. solution of bushy stunt virus was added to a series of tubes each containing 1 ml. of varying dilutions of anti-serum preparations. The inhibition was tested by adding 0·1 ml. of untreated antiserum at a dilution of 1/8.

Symbols as used in Table I.

Antiserum preparation.	Precipitation test. Dilution of antiserum preparations. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	1/128.
1. Unheated antiserum dil. 1/10 . .	+	+	+	+	+	+	+	−
2. Antiserum heated at 75° C. . .	− i	− i	− i	− i	− i	− i	− o	− o
3. Precipitate with 33 per cent. satur. amm. sulph. from (2)	− i	− i	− i	− i	− i	− i	− o	− o
4. Precipitate with 50 per cent. satur. amm. sulph. from the supernatant from (3)	+	+	+	+	− o	− o	− o	− o
5. Precipitate with 100 per cent. satur. amm. sulph. from the supernatant from (4)	− o	− o	− o	− o	− o	− o	− o	− o
6. Antiserum heated at 80° C. . .	− i	− i	− i	− i	− i	− i	− o	− o
7. Sediment from (6) by centrifugation at 15,000 r.p.m.	+	+	− o	− o	− o	− o	− o	− o
8. Supernatant from (7) . . .	− i	− i	− i	− i	− i	− i	− o	− o

TABLE IV.—*The Effect of Heat on the Precipitation Power of Euglobulin Fractions of Antisera.*

0·1 per cent. euglobulin solutions were heated for 15 minutes at 75° C. in the presence and absence of 0·5 per cent. rabbit serum albumin in 0·9 per cent. NaCl at pH 7·0.

Precipitation tests were made as described in Table I.

Treatment of the euglobulin solutions.	Dilution of antiserum preparations. Tobacco mosaic virus. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.	Bushy stunt virus. 1/1.	1/2.	1/4.	1/8.	1/16.	1/32.	1/64.
Unheated . . .	+	+	+	+	+	+	− o	+	+	+	+	+	+	− o
Heated in the absence of albumin	+	+	+	+	+	+	− o	+	+	+	+	+	+	− o
Heated in the presence of albumin	− i	+	+	+	− o	− o	− o	− i	− i	− i	− i	− i	− i	− o
Heated and mixed with albumin heated separately	..	+	+	+	+	+	− o	..	+	+	+	+	− o	− o

TABLE V.—*The Effect of Heat on the Complement-Fixation Power of Euglobulin Fractions of Antisera.*

The solutions used for heating were as described in Table III.
Symbols as in Table II.

Euglobulin solution. Treatment.	Dilution.	Concentration of antigen in 10^{-4} g. per ml. Tobacco mosaic virus. 1·0.	0·3.	0·1.	0·03.	0·01.	0.	Bushy stunt virus. 1·0.	0·3.	0·1.	0·03.	0·01.	0.
Unheated	1/2	++++	++++	++++	++++	+++	−	++++	++++	++++	++++	++	−
	1/4	++	+++	++++	++++	++	−	++++	++++	++++	+++	++	−
Heated in the presence of albumin	1/2	−	+	++	+++	+++	−	++	+++	++++	++++	++	−
	1/4	−	−	−	+	++	−	+	++	++++	++++	+	−
Heated and mixed with albumin heated separately	1/2	++	+++	++++	++++	+++	−	++++	++++	++++	++++	++	−
	1/4	−	+	++	+++	++	−	+++	++++	++++	++++	++	−
	Saline	−	−	−	−	−	−	−	−	−	−	−	−

complement, for after heating for 10 minutes at 80° C. at a dilution 1/10 in saline, the serum did not fix complement at all, although it still combined with globulin to give strong inhibition. They also provide further evidence that fixation of complement is independent of the formation of a precipitate; for after heating at 75° C. the serum also failed to cause precipitation, although it still fixed complement only slightly less strongly than the unheated serum. The difference between the results got with sera heated at the two temperatures can be explained by the different amounts of mixed antibody complexes formed. At 75° C. sufficient were formed to prevent the precipitating antibodies causing precipitation, but insufficient to reduce complement fixation greatly. At 80° C. most or all antibodies were changed to mixed complexes, and complement fixation disappeared. At 90° C. the ability to combine with antigen was quite destroyed and the serum ceased to give the phenomenon of inhibition.

NEUTRALIZATION OF INFECTIVITY.

Experiments on the specific neutralization of infectivity of plant viruses by antisera are greatly complicated by the fact that normal sera and heterologous antisera also reduce infectivity considerably. The specific effect of homologous antisera can be taken as the difference between the reduction in infectivity caused by homologous and other sera. This is far from exact, however, for the unspecific neutralization varies considerably from serum to serum. For this reason, and because of the uncertainties inherent in local lesions tests, the tests we have made on the destruction of specific neutralization power by heat have given less definite results than those on the destruction of abilities to cause precipitation and to fix complement. Nevertheless they show a fairly clear parallelism between the loss of specific neutralization and ability to fix complement.

Table VI gives the results of infectivity tests with tobacco mosaic and bushy stunt viruses mixed with their own and each other's antiserum heated for 10 minutes at various temperatures at a dilution 1/10 in saline. Tobacco mosaic virus was used at 0·001 per cent. and bushy stunt virus at 0·002 per cent.

TABLE VI.—*The Effect of Heat on Neutralization of Infectivity by Antisera.*

The antisera were heated for 10 minutes at a dilution 1/10 in saline. They were then further diluted 1/2 in saline and mixed with equal volume of 0·001 per cent. of tobacco mosaic or 0·002 per cent. of bushy stunt virus solution and tested on *Nicotiana glutinosa*.

Antiserum.	Temperature of heating.	Average number of lesions per leaf with—	
		Tobacco mosaic virus.	Bushy stunt virus.
Homologous	Unheated	24	2
,,	70° C.	16	1
,,	75° C.	25	4
,,	80° C.	39	7
,,	90° C.	41	10
Heterologous	Unheated	31	8
,,	90° C.	31	10
Saline control	..	120	48

1 ml. of saline or 1 ml. of antiserum diluted 1/20 was mixed with 1 ml. of the virus solution, and the mixtures were left for 1 hour at room temperature before being inoculated to *Nicotiana glutinosa*.

It will be seen that with both viruses the heterologous antisera have greatly reduced infectivity, but that the unheated homologous antisera have reduced it still further. After heating at 90° C. the homologous antisera reduce the infectivity no more, and sometimes even less, than the heterologous antisera. Heating at 75° C. reduces the specific neutralizing power slightly, while heating at 80° C. almost destroys it. It will be noted that heating at 90° C. has little or no effect on the unspecific neutralizing action of antisera, although the specific effect is destroyed.

We have also made tests with serum fractions and again got results similar to those in the complement-fixation tests. Euglobulin is much more effective than albumin in reducing the infectivity of virus preparations, and euglobulin from homologous antisera is more effective than that from heterologous sera. When heated alone for 15 minutes at 75° C., 0·1 per cent. solutions of homologous euglobulin lost some of their specific neutralizing power, but when heated in the presence of 0·5 per cent. albumin they lost considerably more. Thus it would seem that mixed complexes are unable to neutralize infectivity.

DISCUSSION.

The fact that antisera to somatic type antigens lose their flocculating power with less heating than do antisera to flagellar type antigens has been generally accepted as evidence that antibodies to the two kinds of antigen differ in their resistance to heat. Kleczkowski (1941) proved this interpretation to be wrong by showing that if euglobulin fractions of the antisera were prepared and heated separately, there was no difference in the amount of heating required to destroy the flocculating power of different antibodies. Only when antibodies to the two antigens were heated in the presence of albumin was there any difference in their behaviour. It was concluded that antibodies could combine with other proteins undergoing heat denaturation simultaneously, and that flagellar antigens are more easily precipitated by mixtures of heat-changed antibodies than somatic antigens. The experiments described in this paper fully support the view that antibodies to the different type antigens are equally affected by heat. When ability to fix complement or to neutralize infectivity are used instead of ability to cause precipitation as tests for residual antibodies, all antisera were found to be equally susceptible to heat. Heating for 10 minutes at 75° C. has little effect on these two serological activities with any serum, while heating at 80° C. almost destroys them with all sera.

Heating at either of these temperatures destroys the ability of antiserum to somatic type antigens to cause precipitation, but not their ability to combine with antigens, for they still have high inhibition titres. Thus, if two samples of an antiserum are heated at these temperatures they behave almost identically in precipitation tests, but one will fix complement strongly and the other not. That the separation of a specific precipitate is not essential for complement fixation is shown by the results of heating antisera to somatic type antigens at 75° C. and also by our results with non-precipitating antigens,

which fix complement with their antisera as strongly as precipitating antigens (Bawden and Kleczkowski, 1942). The explanation of the different behaviour of whole sera when heated at 75° C. and 80° C. is suggested by the results of experiments with mixtures of serum fractions. When euglobulin fractions of antisera are heated alone their ability to fix complement is less reduced than if they are heated with albumin. In other words, heating in conditions permitting the formation of mixed complexes reduces the ability to fix complement. Whether or not the same antibodies are concerned in complement fixation as in precipitation is unknown. If different antibodies are concerned, both kinds must be similarly affected by heat and able to combine with albumin. After combining with albumin, antibodies are unable to fix complement or to cause precipitation. In our tests with heated antisera, complement fixation occurred only when the sera still contained precipitating antibodies. The sera themselves might be unable to cause precipitation, because of the strong inhibitory effect of mixed complexes, but fractions could be isolated from them that did precipitate, and the strength of complement fixation was proportional to the remaining precipitating power of the serum. It is tempting therefore to suggest that the same antibodies are responsible for both serological reactions, and that the ability to fix complement and cause precipitation are closely correlated.

Although precipitation is not essential for complement fixation, something more than mere combination between antigen and antibody seems necessary. Mixed complexes must still combine with antigen, for otherwise their inhibitory action appears inexplicable, yet these do not fix complement. It is possible that the formation of particles of a definite size is necessary for complement to be fixed, and that only antibodies able to cause precipitation produce aggregates of the specific size. If this is so, the presence of mixed complexes can be regarded as preventing this aggregation proceeding far enough to give precipitation, but not preventing the formation of aggregates sufficiently large to fix complement.

SUMMARY.

The effect of heat on serological reactions of antisera to O-type antigens (bushy stunt virus and human serum globulin) and to an H-type antigen (tobacco mosaic virus) were studied.

The complement-fixation power of all antisera, and the specific neutralization of infectivity by virus antisera, were equally affected by heat. Tobacco mosaic virus antisera lost all their serological reactions almost simultaneously, but with O-type antigens less heating was needed to destroy precipitability than to destroy other serological reactions.

These results are explained by the formation of complexes between antibodies and other serum proteins during heating. Mixed complexes, formed with albumin, combine with antigens but are unable to fix complement, neutralize infectivity or cause precipitation ; they inhibit the precipitation of O-type antigens by other antibodies, but do not interfere with complement fixation. Antisera partly denatured by heat contain mixtures of antibodies in different states ; a small proportion of mixed complexes prevents the pre-

cipitation of O-type antigens, but has little effect on complement fixation or on the precipitation of H-type antigens.

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OBSERVATIONS ON THE MOTILITY OF THE REFRACTILE GRANULES OF HUMAN PUS CELLS.

R. E. HOPE SIMPSON.

From the Gable End Laboratory, Beaminster.

Received for publication May 29, 1942.

NEUTROPHIL and eosinophil granulocytes are the cells commonly seen in human purulent matter. They are believed to be derived from similar cells in the blood stream. The granules of the two types show such similarity of behaviour that it is unnecessary for the present purpose to consider them separately after remarking that the granules of the eosinophil are larger and less motile than those of the neutrophil. The refractile cytoplasmic granules of both types exhibit a turbulent motility, more readily studied in the pus cells than in the leucocytes in the blood.

The literature of pus cells, as compared with that of blood leucocytes, is small and scattered. Bergeret (1875), discussing his observations and experiments of 21 years earlier, mentions the motility of the granules of pus cells both inside and outside of the cytoplasmic boundary, and that this motility ceases after 4 days. His description of " pyocytes " clearly includes both neutrophils and eosinophils, and he describes in detail their hygrometric properties.

THEORY THAT GRANULE MOTILITY IS BROWNIAN MOVEMENT.

Garland, in 1879, reporting certain activities shown by pus cells in urine, described the motility of the cytoplasmic granules. During the subsequent discussion Dwight suggested that the granule motility was probably " Brunonian movement in the protoplasm of the cell." The suggestion was favourably received by the meeting and has since gained wide acceptance. The cytoplasm is considered by some to be a colloidal material capable of undergoing reversible changes from sol to gel. The conception may be outlined as follows : In the sol phase the granules exhibit Brownian movement, and in the gel phase the movement is arrested. These changes can occur in localized areas of the cytoplasm, and they are believed to provide a mechanism for amoeboid movement. At death of the cell irreversible gelation of the cytoplasm is said to occur. Evidence for the validity of this conception is alleged to be seen in

the facts that many granulocytes contain areas of stationary and areas of motile granules in the cytoplasm, and that in the dead cell the granules lie in a motionless clump around the nucleus. Closer observation of the granules does not, however, support this conception of the cytoplasm, and casts doubt on the assumption that the granules owe their turbulence to Brownian movement.

GENERAL OBSERVATIONS.

(*a*) In its resting phase in fresh unstained purulent material the pus cell is spherical with a near-central *spherical* nucleus, of which the lobations can often be discerned as faint lines like the seams on a football. The cytoplasm is packed with granules all in a state of violent turbulence. They resemble the seething bubbles on a parboiling pot. An early French observer used the verb " fourmiller " to describe this appearance. Such cells are well seen in sputum from young asthmatics, pus from mild infection, and sputum from tuberculosis during remissions.

The motility is so great that it suggests some motive power other than Brownian movement, unless molecular activity within the cytoplasm is of a peculiar order.

(*b*) The granules can be observed to pass freely through the external cytoplasmic boundary, each thus becoming the tip of a very slender process of cytoplasm. These processes exhibit a vigorous lashing movement of a violence and speed resembling the motility of the intracytoplasmic granules. The whole cell may become covered with a wavy coat of such " tentacles," each extruded to a distance of about 2μ. These are easiest to watch in thin sero-pus or purulent urine.

(*c*) These processes or tentacles can be readily withdrawn within the cytoplasmic boundary, or some may lengthen, often extending to relatively great distances, 100μ or more, from the parent cell. In such filopodia the distal end, for several μ from the tip, retains the power of waving motility. Other granule-tipped tentacles tend to follow the same path until a relatively thick strand is formed. A granule passing along such a strand from the cell may leave it part way, as the tip of a branch taking an independent path. Further details of these phenomena cannot be given here, and they are mentioned because they must be taken into account in any discussion on the nature of granule motility.

(*d*) Pus cells, e.g. those in sputum from acute febrile catarrhs, often have nuclei which are widely lobate, and cytoplasm very incompletely filled with granules, of which those in some areas of the cytoplasm are motionless.

(*e*) *Gradual cell death.*—In fresh preparations most pus cells are dead or moribund on the third day. The cells which have died gradually have an empty cytoplasmic space, occupying about two-thirds of the cell, the remainder containing the nucleus at the periphery surrounded by the motionless granules, now shrunk into a little cluster. The moribund cells resemble the dead, with one difference of theoretical importance. The moribund cells contain a few granules dancing freely around the otherwise empty cytoplasmic space. Watch such a cell with about six motile granules. It will be seen that one by one the granules drop out of the dance and join their motionless fellows around

the nucleus, until penultimately one only is left, with the space to itself. Still there is no limitation of its movement, and it can range freely in all the available cytoplasmic space before it, too, joins the group. This shows clearly that, whatever the nature of the cytoplasm, and whatever the changes taking place at the death of the cell, the immobilization of the granules is not in this instance evidence of irreversible gelation of the cytoplasm.

(*f*) In sputum, Brownian movement of particles in the slime containing the cells is seldom evident. Nevertheless cytoplasmic granules on filopodia from the cells (*cf.* paragraph *b*) often show vigorous movement, while free particles of a similar size, which abound in the matrix, are motionless.

The above observations require minimal interference with the natural circumstances of the fresh material.

SPECIAL OBSERVATIONS INVOLVING MANIPULATION OF THE MATERIAL.

(*a*) Methylene blue acts as a fairly rapid and fatal poison to pus cells. When 1 per cent. watery Methylene Blue is allowed to be drawn under the cover of a live preparation, the nuclei of dead cells immediately take the stain and become intensely blue, the cytoplasm harbouring the stain in the same degree as the circumambient fluid. The living cells, on the contrary, resist the stain and stand out as bright uncoloured refractile bodies against the blue background, much like moons in a night sky, and the turbulence of their granules continues.

For 10 or 15 minutes such a cell may continue to resist. At length a faint tinge of blue appears in the nucleus and in a few large granules, though the cytoplasm and most of its granules remain colourless and refractile. The end comes with dramatic suddenness. The violent activity of the granules ceases abruptly, most of them seem to disappear and the remainder clump irregularly. At the same moment the stain sweeps into the cell, darkens the nucleus, colours the cytoplasm to the same depth as the surrounding medium and leaves the cell with no refractility.

(*b*) Should the cells in such a preparation have granules externally deployed in tentacles as described, the activity of such external granules also ceases when the parent cells are killed by the methylene blue. This seems to me powerful evidence against Brownian movement as the cause of motility.

CONCLUSIONS.

The above observations suggest —

(1) That the motility of the refractile cytoplasmic granules of pus cells is not due to Brownian movement.

(2) That the behaviour of the cytoplasmic granules cannot be taken as evidence in favour of colloidal cytoplasm with reversible sol-gel potentialities.

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THE INCIDENCE OF PRIMARY LUNG TUMOURS IN A MIXED STRAIN OF MICE.

J. ARGYLL CAMPBELL.

From the National Institute for Medical Research, London.

Received for publication June 9, 1942.

DURING the past ten years a series of researches has been conducted at this Institute dealing with the effects of inhalation of various dusts and gases upon the incidence of primary lung tumours in the descendants of a mixed strain of mice originally obtained in the open market. A good deal of information has been now obtained with regard to the incidence of primary lung tumours in chocolate, fawn and white mice. Since similar mice are still being used here for experiments and since various other laboratories have requested and been granted supplies of these mice, it is considered that this statistical information, which affords a base-line for future research, should be made generally available.

It may be pointed out that there is no close connection between the present mice and those used by Sir Leonard Hill (1931) in his research on the effect of various diets on spontaneous mouse tumours in general. In 1926 he selected at random 44 males and 44 females from the common breeding stock of the mixed strain of this Institute and from these bred 793 mice. In all he obtained 140 of these mice with lung tumours—that is, an incidence of 17·6 per cent. On the other hand the mice used in the present research were bred at the Medical Research Council's Farm at Mill Hill. Some of their ancestors were originally obtained by the late Major G. W. Dunkin, but more recently the breeding of the mice has been under the supervision of Dr. R. E. Glover, to whom I am indebted for data regarding the origin of the mice and their handling during the past ten years. The parent stock was a mixed strain of market mice of unknown origin. Beyond eliminating all black and black-and-white mice and concentrating on the chocolate (brown), fawn and white mice, no attempt has been made at selection. There has been no brother to sister mating. About 1936 a strain of black-and-white mice was brought to the Farm and a certain percentage of Farm does crossed with black-and-white bucks. Soon, however, the policy of introducing black-and-white blood was stopped, all mice showing this colour being killed at birth. By 1939 the tendency to throw such dark-coloured animals was practically eliminated. Of course some other character may have been introduced by this black-and-white strain and become now permanently fixed in the stock. I, however, have found no clear evidence in these experiments of such a factor connected with the incidence of primary lung tumours. Since 1934 there has been no other introduction of outside mice, but there is in use at the Farm another inbred strain of white

mice which is kept separate from the above mixed strain. On rare occasions when white mice of the mixed strain were not sufficiently numerous, some small numbers of these inbred mice were issued for experiment along with the mixed strain. These few special inbred white mice did not appear to behave very differently with regard to the incidence of lung tumours.

The colours of the mice of this mixed strain included chocolate, white, fawn, ginger, black, grey and various mixtures of these colours ; for example, fawn and white, black and white, and so on. The great majority of the mice used, however, were either chocolate, white or fawn, and attention is directed to these three groups only since the numbers of the other colours were few.

TABLE I.—*Distribution of 2225 Mice at Start of Experiments.*

Colour.	Male (M) or female (F).	986 Controls (C). No.	986 Controls (C). %	1239 Experimental (E). No.	1239 Experimental (E). %	2225 C + E. No.	2225 C + E. %
Chocolate	M.	116	11·8	160	12·9	276	12·4
	F.	129	13·1	160	12·9	289	13·0
	M. + F.	245	24·9	320	25·8	565	25·4
White	M.	165	16·7	183	14·8	348	15·6
	F.	161	16·3	200	16·1	361	16·2
	M. + F.	326	33·0	383	30·9	709	31·8
Fawn	M.	206	20·9	251	20·3	457	20·5
	F.	209	21·2	285	23·0	494	22·2
	M. + F.	415	42·1	536	43·3	951	42·7

Distribution of the mice at the start of the experiments.—The total number of mice considered herein is 2225, consisting of 986 control and 1239 experimental mice. For an experiment normally 150 mice about three months old were employed, half of them male and half female. In the great majority of experiments the sexes were kept separate, and in only a few of the earlier researches involving the inhibitory effects of carbon monoxide upon fertility were a certain number of boxes kept containing one male with several females. Of the 150 mice, 75 were used for the control group and 75 for the group exposed to the dust or gas. Occasionally one batch of control mice was used for two separate experiments started at the same time ; thus there were then 225 mice in all, 75 in the control group, and two groups of experimental mice also 75 each in number. This explains why on the whole there were more experimental than control mice. In selecting the mice attempt was made to place equal numbers of the same colours, in each batch of 75 mice, both control and experimental.

Of the total 2225 mice, 565 or 25 per cent. were chocolate, 709 or 32 per cent. white, and 951 or 43 per cent. fawn (Table I). The endeavour to have equal numbers of male and female mice was nearly successful, since of the total 48·6 per cent. were male and 51·4 per cent. female. In both control and experimental groups there were more fawn mice than white and more white than chocolate. Of the 986 control mice 245 or 25 per cent. were chocolate, 326

or 33 per cent. white, and 415 or 42 per cent. fawn. Very similarly as to percentages there were among the 1239 experimental mice 320 or 26 per cent. chocolate, 383 or 31 per cent. white, and 536 or 43 per cent. fawn. Therefore the composition of the total control and experimental groups was, as Table I shows, virtually identical in respect of both colour and sex.

TABLE II.—*Distribution of* 1739 *Mice Living Ten Months or Longer.*

Colour.	Male (M) or Female (F).	799 Controls (C). No.	799 Controls (C). As % of No. at start of expt.	940 Experimental (E). No.	940 Experimental (E). As % of No. at start of expt.	1739 C + E. No.	1739 C + E. As % of No. at start of expt.
Chocolate	M.	89	77	112	70	201	73
	F.	116	90	132	83	248	86
	M. + F.	205	84	244	76	449	79
White	M.	122	74	138	75	260	75
	F.	136	84	156	78	292	81
	M. + F.	258	79	294	77	552	78
Fawn	M.	146	71	171	68	317	69
	F.	190	91	231	81	421	85
	M. + F.	336	81	402	75	738	78
Totals	M.	357	73	421	71	778	72
	F.	442	89	519	80	961	84
	M. + F.	799	81	940	76	1739	78

Distribution of the mice at ten months of age.—In the various papers published it has been clearly demonstrated that primary lung tumours are very rare indeed in this mixed strain of mice before ten months of age. Therefore the distribution of mice at this stage is important and is shown in Table II. Of the original 2225 mice, 1739 or a little more than 75 per cent. lived for ten months or longer, 799 with an 81 per cent. survival rate, control mice, and 940, with a 76 per cent. survival rate, experimental mice. It will be seen that in all groups there were distinctly more female than male mice alive at this stage, in fact 84 per cent. of the total female mice compared with 72 per cent. of the total male mice. This is due to the fact that the male mice even when separated from the females fight among themselves more frequently and more vigorously than do the females, and thus the less aggressive male mice are killed off earlier.

It will be noted from Table II that no significant differences are to be seen in the survival rates of mice of different colours, the total figures being 79 per cent. for chocolate, 78 per cent. both for white and fawn. On the other hand the experimental procedure seems to have had some effect on the early mortality, since of the total control mice 81 per cent. were still alive compared with 76 per cent. of the experimental group after seven months' exposure to the dusts. This difference is unlikely to be due to chance, since not only is it nearly three times its standard error ($5 \pm 1{\cdot}76$), but it is apparent in five of the six sex and colour sub-groups.

TABLE III.—*Distribution of* 362 *Lung Tumours and* 10 *Metastases.*

Colour.	Male (M) or Female (F)	90 controls with tumours.			272 experimental mice with tumours.		
		Simple.	Malignant.	Metastases.	Simple.	Malignant.	Metastases.
Chocolate	M.	4	4	0	8	9	0
	F.	7	5	0	16	23	1
	M. + F.	11	9	0	24	32	1
White	M.	10	3	0	13	16	0
	F.	7	7	0	25	26	3
	M. + F.	17	10	0	38	42	3
Fawn	M.	9	7	0	23	28	0
	F.	12	15	1	33	52	5
	M. + F.	21	22	1	56	80	5
Totals	M.	23	14	0	44	53	0
	F.	26	27	1	74	101	9
	M. + F.	49	41	1	118	154	9

Distribution of 362 *primary lung tumours and* 10 *metastases.*—In all there were 362 mice with primary lung tumours, and of these 90 were control mice and 272 experimental. In 49 of the 90 control mice the tumours were simple, while the remaining 41 or 45·6 per cent. possessed malignant tumours. Among the 272 experimental mice with lung tumours, 118 had simple tumours, while 154 or 56·5 per cent. revealed malignant tumours. The dusting thus increased the incidence of malignancy to some degree. Further there was only one case

TABLE IV.—*Percentages of the Mice with Tumours among Mice Living* 10 *Months or Longer.*

Colour.	Male (M) or Female (F).	Controls.			Experimental.		
		Simple (S).	Malignant (M).	S + M.	Simple (S).	Malignant (M).	S + M.
Chocolate	M.	4·5	4·5	8·9	7·1	8·0	15·2
	F.	6·0	4·3	10·3	12·1	17·4	29·5
	M. + F.	5·4	4·4	9·8	9·8	13·1	22·9
White	M.	8·2	2·5	10·6	9·4	11·6	21·0
	F.	5·1	5·1	10·3	16·0	16·6	32·7
	M. + F.	6·6	3·9	10·5	12·9	14·3	27·2
Fawn	M.	6·2	4·8	10·9	13·5	16·4	29·8
	F.	6·3	7·9	14·2	14·3	22·5	36·8
	M. + F.	6·3	6·5	12·8	13·9	19·9	33·8
Totals	M.	6·4	3·9	10·4	10·5	12·6	23·0
	F.	5·9	6·1	12·0	14·3	19·5	33·7
	M. + F.	6·0	5·1	11·3	12·6	16·4	28·9

of a metastasis, or extension of the primary lung tumours beyond the lung, among the control mice giving an incidence of 1·1 per cent., whereas there were 9 such extensions in the experimental group, an incidence of 3·3 per cent. All the metastases were in female mice, which also usually showed a greater number of mice with tumours than did the males. As will be seen later these differences are due to the fact that more females than males survived to the older ages.

Influence of sex.—When the mice with tumours are expressed as a percentage of the mice living ten months or longer, the males and females amongst the controls do not show any great difference in incidence; thus of the total males 10·4 per cent. and of the females 12·0 per cent. possessed tumours (Table IV). For all three colours, chocolate, white and fawn, there are about 10 per cent. of both male and female mice with either simple or malignant tumours, the only group showing any appreciable difference being the female fawn mice with 14·2 per cent. On the other hand among the experimental mice the females in all cases show a higher incidence of lung tumours than the corresponding males; thus of the total 421 males 23·0 per cent. revealed lung tumours compared with 33·7 per cent. of the total 519 females. Here again the female fawn mice show the highest incidence, namely 36·8 per cent.

To investigate further this difference in tumour incidence between male and female mice, the percentage tumour incidence in mice dying during 5-monthly periods, namely 10 to 15 months of age, 15 to 20, 20 to 25, and so on, is given in Table V. As regards the controls there is no striking difference between the 15 comparable incidences for males and for females over these 5-monthly periods. There are 9 incidences where the mice totalled for each sex more than 20, and in 7 of these the males had the higher incidence. In the case of the experimental mice in 10 of the 15 incidences the females had the higher figure, but taking only those 9 groups where the mice totalled more than 20 for each sex separately, it is noted that in 8 of these 9 groups the females had the higher incidence, so that the position is just about the reverse of that for the controls.

TABLE V.—*Percentage Tumour (T) Incidence in Mice (N) Dying during 5-monthly Periods.*

Mice.	Colour.	10–15 months.		15–20 months.		20–25 months.		25–30 months.		30–35 months.		Total.	
		T/N.	%.	T/N.	%.	T/N.	%.	T/N.	%.	T/N.	%.	T/N.	%.
Control males	Chocolate	0/32	0·0	1/26	3·8	4/21	19·0	3/10	30·0	—	—	8/89	8·9
	White	2/44	4·5	3/33	9·1	5/28	17·9	3/16	18·5	0/1	0·0	13/122	10·6
	Fawn	0/49	0·0	3/36	8·3	8/49	16·3	4/10	40·0	1/2	50·0	16/146	10·9
Control females	Chocolate	1/27	3·7	1/32	3·1	6/43	13·9	3/12	25·0	1/2	50·0	12/116	10·3
	White	0/32	0·0	3/43	7·0	3/34	8·8	8/26	30·8	0/1	0·0	14/136	10·3
	Fawn	1/30	3·3	3/61	5·0	9/59	15·3	10/35	28·6	4/5	80·0	27/190	14·2
Experimental males	Chocolate	1/33	3·0	5/40	12·5	7/30	23·3	4/8	50·0	0/1	0·0	17/112	15·2
	White	2/41	4·9	9/48	18·8	12/40	30·0	4/7	57·1	2/2	100·0	29/138	21·0
	Fawn	3/52	5·9	19/59	32·2	22/47	46·8	4/10	40·0	3/3	100·0	51/171	29·8
Experimental females	Chocolate	1/32	3·1	8/39	20·5	13/32	40·6	11/23	47·8	6/6	100·0	39/132	29·5
	White	3/30	10·0	13/43	30·2	16/41	39·2	19/39	48·7	0/3	0·0	51/156	32·7
	Fawn	0/48	0·0	24/69	34·8	37/72	51·4	21/37	56·8	3/5	60·0	85/231	36·8

TABLE VI.—*Mice Dying during 5-monthly Periods Expressed as a Percentage of the Number Alive at 10 months.*

Mice.	Colour.	10–15 months.	15–20 months.	20–25 months.	25–30 months.	30–35 months.
Control males	Chocolate	36·0	29·2	23·6	11·2	—
	White	36·1	27·0	22·9	13·3	0·8
	Fawn	33·6	24·7	33·6	6·8	1·3
Control females	Chocolate	23·3	27·6	37·1	10·3	1·7
	White	23·5	31·6	25·0	19·1	0·8
	Fawn	15·8	32·1	31·1	18·4	2·6
Experimental males	Chocolate	29·5	35·7	26·8	7·1	0·9
	White	29·7	34·8	28·9	5·1	1·5
	Fawn	30·4	34·5	27·5	5·8	1·8
Experimental females	Chocolate	24·2	29·6	24·2	17·4	4·6
	White	19·2	27·6	26·2	25·0	2·0
	Fawn	20·8	29·9	31·2	16·0	2·1

Influence of age.—The reason why, for the total numbers of mice, the females reveal the higher incidence of lung tumours is that relatively and absolutely more females than males lived to more than 25 months of age. This is demonstrated in Table VI, where the mice dying during 5-monthly periods are expressed as a percentage of the number alive at 10 months. It is also clear from Table V that for each colour and sex the tumour incidence increases with age. At the highest age period, namely 30 to 35 months, although some of the groups of very few mice show an incidence of *nil*, this is counterbalanced by other groups with an incidence of 100 per cent.

TABLE VII.—*Influence of Colour. All Mice taken together.*

Colour of mice.	Percentage incidence of lung tumours.		
	Control (C).	Experiment (E).	E/C.
Chocolate	9·8	22·9	2·34
White	10·5	27·2	2·59
Fawn	12·8	33·8	2·64

Influence of colour.—Returning to Table IV there is a suggestion that susceptibility to tumours runs in the order of colour, chocolate, white and fawn, with the fawn mice having the highest incidence ; this appears to hold for both male and female mice judging from the figures for simple and malignant tumours taken together. If, to get larger figures, the male and female results are taken together, the position is as in Table VII. Not only do the figures for the controls run in the same order as those for the experimental, but the differences are somewhat increased with dusting, suggesting increasing liability to experimental tumour development. On the other hand the figures for X^2 for the four groups, male, female, control and experimental, as arranged in the columns S and M, Table IV, give a significant result only for the experimental males, and from this point of view the other increases with colour in the order

TABLE VIII.—*Colour and Sex of Mice and Incidence of Tumours of Lung. Various Batches of Mice Living 10 months or Longer.*

Dates of batches.	Chocolate. Males.	Chocolate. Females.	White. Males.	White. Females.	Fawn. Males.	Fawn. Females.	Totals for all three colours. Males + Females.
Controls :							
1932–1935 .	1/9 =11·1%	2/14=14·3%	1/8 =12·5%	2/23= 8·7%	5/35=14·3%	5/46 =10·9%	16/135=11·9%
1933–1936 .	2/15=13·3%	4/14=28·6%	3/20=15·0%	4/17=23·5%	1/23= 4·3%	10/42=23·8%	24/131=18·3%
1934–1937 .	1/4 =25·0%	0/6 = 0·0%	2/8 =25·0%	1/6 =16·6%	4/16=25·0%	5/14 =35·7%	13/54 =24·1%
1937–1939 .	3/21=14·3%	4/24=16·6%	3/28=10·7%	2/30= 6·6%	3/25=12·0%	1/32 = 3·1%	16/160=10·0%
1938–1940 .	1/40= 2·5%	2/58= 3·4%	4/58= 6·9%	5/60= 8·3%	3/47= 6·4%	5/56 =10·7%	21/319= 6·6%
Totals .	8/89=8·9%	12/116=10·3%	13/122=10·6%	14/136=10·3%	16/146=10·9%	27/190=14·2%	90/799=11·3%
Experiments :							
1932–1935 .	6/15=40·0%	10/22=45·4%	12/21=57·1%	14/28=50·0%	17/36=46·9%	39/74=52·7%	98/196=50·0%
1933–1936 .	3/14=21·4%	2/16=12·5%	2/18=11·1%	2/14=14·3%	11/32=34·4%	14/43=32·6%	34/137=24·8%
1934–1937 .	2/9 =22·2%	4/12=33·3%	2/13=15·4%	3/11=27·3%	8/22=36·4%	6/19=31·6%	25/ 86=29·1%
1937–1939 .	2/32= 6·2%	16/35=45·7%	2/26= 7·7%	11/40=27·5%	10/32=31·2%	14/42=33·3%	55/207=26·6%
1938–1940 .	4/42=9·5%	7/47=14·9%	11/60=18·3%	21/63=33·5%	5/49=10·2%	12/53=22·6%	60/314=19·1%
Totals .	17/112=15·2%	39/132=29·5%	29/138=21·0%	51/156=32·7%	51/171=29·8%	85/231=36·8%	272/940=28·9%
Controls + experiments	25/201=12·4%	51/248=20·6%	42/260=16·2%	65/292=22·1%	67/317=21·1%	112/421=26·6%	362/1739=20·8%

chocolate, white, fawn, might easily have been due to chance. Nevertheless the consistency does suggest a real, but slight, increase in susceptibility in this order.

The question, however, must also be considered from the point of view of the relative numbers of mice of different colours. In Table VIII all the experiments are arranged in five periods dating from 1932 to 1940. If the male plus female rates for all colours at the right-hand end of this table are taken to calculate how many lung tumours are to be expected in the chocolate, white and fawn groups at those rates in each period and the results added up for all periods, the figures in Table IX are obtained. Although there does not seem to be much difference, there is still a suggestion that the fawn mice have been more susceptible to development of lung tumours.

TABLE IX.—*Numbers of Observed and Expected Lung Tumours for all Mice in the Three Colours.*

Rate.	Control.			Experimental.		
	Chocolate.	White.	Fawn.	Chocolate.	White.	Fawn.
Expected .	21·4	27·4	41·3	66·9	80·5	124·7
Observed .	20	27	43	56	80	136

Influence of exposure to the dusts.—There is no doubt that taking all the experiments together the exposure to the dusts increases the incidence of lung tumours in mice. This increase is due chiefly to the three series of experiments included in the years 1932–1935, 1937–1939, and 1938–1940, and the incidences for lung tumours in these batches were much above those for the corresponding controls (Table VIII). Indeed these increases are not only obvious for each colour and sex, but also go far beyond what might be expected to occur by chance, the figures for X^2 being 48·8, 14·8 and 21·4 respectively, and for P far less than 0·01. The group 1932–1935 contains the experiments with tarred road dust (Campbell, 1934), and those with the tar-free dust (Campbell, 1937), where there were marked increases in incidence of lung tumours as the result of exposure to the dusts. The group 1937–1939 includes some experiments with brown oxide of iron and precipitated silica where there were also increased incidences (Campbell, 1940). The group 1938–1940 contains experiments with Czechoslovak dust and further experiments with iron oxide and silica wherein some increase in incidence was also observed (Campbell, 1942). The other two groups 1933–1936 and 1934–1937 show slight increases (Campbell, 1936 ; 1938), for which the figures for X^2 are 1·3 and 0·2 respectively, so that the increases here may have been due to chance.

Recent tendency for incidence in the controls to fall.—Since 1932 the incidence of lung tumours among the control mice has for some unknown reason undergone some variations (Tables VIII and X) which emphasize the necessity for each experimental batch of mice to be controlled by a similar batch of control mice, that is, the figures for the control mice for one year should not be taken as suitable for controlling an experiment in another year. Nevertheless the increases due to the dusting would in most cases be still there, even if the highest incidence among the control groups were taken as a base line.

TABLE X.—*Incidence of Primary Lung Tumours in the Control Groups.*

Dates of batches.	Percentages of mice with lung tumours.	
	On number of mice at start of expt.	On number of mice living 10 months or longer.
1932–1935	9·4	11·9
1933–1936	13·7	18·3
1934–1937	21·3	24·1
1937–1939	8·2	10·0
1938–1940	5·5	6·6
Total	9·1	11·3

More recently (1938–1940) the incidence in the control mice has shown a tendency to fall. This fall seems to have affected both the males and females among the chocolate mice, but only the males among the white and fawn. This fall was preceded by a rise in incidence among the 1933–1936 and 1934–1937 batches. Here the females were affected as well as the males. For all the mice taken together the incidence was 11·3 per cent. for mice living ten months or longer and 9·1 per cent. for all mice from three months of age (Table X).

Incidence of mammary and other tumours.—Some figures are available for the incidence of mammary tumours. Since breeding was almost negligible, being allowed only amongst a few of the mice, the low incidence, 1·9 per cent. for all 1739 mice, is to be expected. The control mice showed an incidence of 2·5 per cent. compared with 1·4 per cent. for the experimental mice; and among the controls the female chocolate mice had the highest incidence, 6·9 per cent., while the incidences for the female white and female fawn mice were 2·9 and 3·7 per cent. respectively. There were no striking differences for colour, and of the total 33 tumours only two were in males. There were isolated examples of tumours of other organs, for example adenoma of the liver, while the only tissue, apart from the lung and mamma, to show tumours in any number was the lymphoid tissue. Data are given in the various publications for the influence of the dust upon the lymphoid tissue of the lung and the tracheobronchial lymph glands. Some data are also given in these papers for lymphosarcoma and lymphadenoma. Amongst all the mice living about ten months or longer there were 58, or 3·3 per cent., with some such type of lymphoid tumour, the incidences for the males and females being 3·0 and 3·6 per cent. respectively. The chocolate mice with 4·4 per cent. showed the highest incidence for colour with 3·4 per cent. for the white mice and 2·6 for the fawn. The incidences among the control and experimental groups were similar, being 3·2 and 3·5 respectively. The highest incidence of all was 5·4 per cent. for the experimental male chocolate mice, with the control female chocolate mice second with 5·2. The lowest incidence was 0·7 per cent. for the experimental male white mice, and the control female fawn mice also had a low incidence, namely 1·6 per cent. The leukaemias have not been included in the above figures for lymphoid tumours.

DISCUSSION.

Some observers have expressed the opinion that for experimental work on cancer pure strains of inbred mice should be employed. There is no doubt that such a condition should simplify the factors involved. Nevertheless it seems obvious that experiments with mixed strains should not be banned at this stage, since cancer as seen in man occurs in mixed strains. Indeed the only situation where some degree of inbreeding may be exercising its influence in lung cancers seems to be the Czechoslovak pitchblende mines. Prof. J. Löwy informs me that the mines were opened there in the year 1516 as silver mines and the name Thaler and the translation " Dollar " come from Joachimsthal. Since that time Prof. Löwy states the descendants of the same families, or nearly the same families, have worked in the mines ; the son followed always the father in the occupation and the families married with each other. This seems to be the best known example of inbreeding connected with human lung cancer. Heredity, in addition to the radium and arsenic, may certainly play an important part in producing lung cancer among these miners since, by inbreeding, pure strains of mice have been obtained with nearly 100 per cent. incidence of spontaneous lung tumour, while in other pure strains the incidence is very low indeed. More recently also variations in incidence of mammary cancer seem to be occurring even in these pure inbred strains as they are carried on further (Burrows, 1941), and it is too early therefore to advocate exclusion of mixed strains from experiments, especially as the base lines, including those obtained in the present researches, seem sufficiently constant for conclusions to be reasonably drawn.

In human lung cancer males appear to be affected much more often than females, but Brunn and Goldman (1941) state that for simple bronchial adenoma the reverse is the case. With the mice on the whole sex does not appear to play any great part, but in the case of the mice both the male and female were exposed equally to the adverse experimental conditions. In man environmental conditions are usually different for the two sexes for a great part of the day.

SUMMARY AND CONCLUSIONS.

(1) The incidence of lung tumours in a mixed strain of mice, used for research during the past ten years (1932–1941), has been studied statistically in 2225 mice aged three months or older and of colour either chocolate, white or fawn.

(2) Of these mice, 1739 or about 78 per cent. lived 10 months or longer. The sexes were kept separate as a rule, yet the early mortality was higher among the males than the females owing to the males fighting. Colour had no influence on mortality, but the experimental procedure increased the early mortality.

(3) Lung tumours are very rare indeed in this mixed strain of mice before 10 months of age, and the facts below refer only to mice exceeding this age.

(4) Of the 1739 mice, 799 were kept as control mice and 940 were exposed

to various dusts chiefly, but in some experiments the mice were exposed to gases. Taking both control and experimental mice together, 449 were chocolate, 552 white, and 738 fawn. The relative distributions of sex and of colour were even amongst the control and experimental groups.

(5) The incidence of primary lung tumours amongst all the control mice was 11·3 per cent. Variations were observed from year to year so that each experiment should be controlled by control mice of the same age and batch.

(6) There seems to be a real but slight increase in liability to development of lung tumours in the colour order chocolate, white, fawn, with the fawn mice having the highest incidence. This is seen in both the control and experimental groups of mice.

(7) Sex in itself has no great influence on the incidence of lung tumours, but the female fawn mice had the highest incidence. The males and females were, of course, equally exposed to the experimental conditions.

(8) Age is a most important factor, since for both sexes and all colours the incidence of lung tumours increases with age. The female mice possessed tumours in greater numbers and with greater malignancy than the males, because more females than males survived to the older ages.

(9) Certain dusts—tarred road dust with and without tar ; Czechoslovak pitchblende dust ; silica dust ; brown oxide of iron—increase the incidence of lung tumours in mice beyond the limit that includes chance.

(10) The incidence of mammary tumours amongst all the mice, control and experimental, was 1·9 per cent. ; that for tumours of lymphoid tissue, excluding the leukaemias, was 3·3 per cent.

I am indebted to Dr. A. Bradford Hill for advice and help with the statistical examination ; also to Mr. C. Pergande, my technical assistant, and to Mr. F. J. Higginson for cutting and staining the sections.

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AN ANTIBACTERIAL SUBSTANCE PRODUCED BY *PENICILLIUM CLAVIFORME.*

E. CHAIN,* H. W. FLOREY AND M. A. JENNINGS.*

With the Technical Assistance of

D. CALLOW.

From the Sir William Dunn School of Pathology, University of Oxford.

Received for publication June 13, 1942.

WILKINS and Harris (1942) found that *Penicillium claviforme* when grown in certain culture media produced one or more substances which inhibited the growth of the test organisms *Staph. aureus*, *Bact. coli* and *Ps. pyocyanea*. The following paper contains a description of the chemical and biological properties of an antibacterial substance which has been isolated in a crystalline form, for which the name claviformin is proposed.

EXPERIMENTAL.

The culture medium containing the substance was supplied by Wilkins and Harris. It was reduced to 1/20 of its original volume by evaporation at pH 5 *in vacuo*, the water bath temperature being 45° C. The concentrated solution was extracted 8 times with one-fourth of its volume of ethyl acetate. The ethyl acetate solution was evaporated to dryness, leaving a red to dark brown semi-solid residue. This was extracted several times with 50 c.c. portions of chloroform. The residue (A) remaining after these extractions was a dark tar-like material from which a certain amount of impure crystalline antibacterial substance was obtained by the method described below. The chloroform extract was evaporated *in vacuo* to dryness and the residue left in an evacuated desiccator over P_2O_5. Usually crystallization had begun after 24–48 hours. At the end of this time 20–30 c.c. of chloroform were added and the material was warmed. On cooling to room temperature and rubbing the walls of the vessel with a glass rod crystallization began. Some hours later the crystals were removed by filtration. From the mother liquor a second crop of crystals was obtained after concentration. On further concentration small amounts of another crystalline substance separated in needles which gave a strong violet colour with ferric chloride; this did not possess any antibacterial action. The combined crystalline antibacterial substance (yield 4 gm. from 12·5 l. of culture medium) was extracted twice with approximately 2 parts of cold

* With personal grants from the Medical Research Council.

chloroform, which removed brown oily impurities. It was then recrystallized from hot chloroform. The substance was not very soluble even in hot chloroform, and for 200 mg. of crude material 5–6 extractions with portions of 1 c.c. of hot chloroform were necessary. The chloroform extract was then concentrated, and on standing the substance crystallized in colourless rhomboid plates. A further yield of crystalline substance could be obtained from residue A. This was extracted with chloroform in a Soxhlet apparatus. A black, almost solid tar which separated during the extraction was discarded. The chloroform extract, from which a certain amount of brown oily material separated, was left standing for several days and the chloroform was allowed to evaporate slowly during this period. Gradually crystallization began and big crystals (up to 4 mm.^{2}) separated. They were extracted several times with cold ether, which removed impurities and recrystallized from hot chloroform or a mixture of benzene and methyl alcohol (4 : 1).

Chemical Properties.

Melting point.—The melting point of a sample recrystallized three times from chloroform was 110° C. It did not rise after a further recrystallization. The substance sublimed slowly at between 80 and 100° C. in a high vacuum.

Solubility.—The substance was fairly soluble in water, very soluble in alcohol and acetone, moderately soluble in ether and insoluble in benzene and petroleum ether.

Elementary analysis (*Weiler*).—C : 54·2 per cent. ; H : 31·8 per cent. ; active hydrogen (Zerewitinoff in pyridine) : 0·5 per cent. Nitrogen was absent. The preparation contained 1 per cent. of sulphur and a trace of chloride ; these were no doubt impurities which were still present even after several recrystallizations from chloroform. From the analytical figures the most probable elementary formula is $C_9H_8O_5$ (C : found 54·2 per cent., calculated 55·1 per cent. ; H : found 3·8 per cent., calculated 4·0 per cent.). A molecular weight determination by the osmotic method of Barger and Rast gave a value of 182 (calculated 196). The low value was presumably due to the presence of low molecular impurities. The figure for active hydrogen corresponded to one atom per molecule.

Rotation.—A 1 per cent. solution showed no optical activity.

Reaction in water.—A 1 per cent. solution in water gave a pH value of 6·5.

Reducing power.—The substance possessed a strong reducing power. Ammoniacal silver nitrate and Fehling's solution were reduced in the cold. With 2 : 4 dinitrophenylhydrazine in 2N. H_2SO_4 a yellow crystalline precipitate started to form after 15 minutes.

Stability at different pH values.—The antibacterial activity of the substance remained unimpaired in acid solution even after boiling in N/10 HCl for 30 minutes. In alkaline solution the activity quickly disappeared ; at pH 10 over 90 per cent. of the activity was lost after incubation at 37° for 1 hour and at pH 9 about 50 per cent. Boiling at pH 7 for 10 minutes destroyed most of the activity. In alkaline watery solution a reddish colour developed gradually and was pronounced after 24 hours.

Action of ammonia.—A yellow colour developed after the addition of dilute

ammonia to a dilute watery solution. It was pronounced at a concentration of 1 : 1000 and perceptible at 1 : 50,000. This colour was quite different from the reddish colour obtained with alkali. Piperidine, asparagine and amino acids in alkaline solution produced the same colour, as also did peptone culture media. The development of the yellow colour did not affect the antibacterial activity of the substance.

The crystalline antibacterial substance isolated from culture medium of *P. claviforme* accounts for most of the antibacterial power. It is possible, however, that in addition, derivatives, especially the combination with NH_3 and other bases, are also present.

Biological Properties.

Titration of antibacterial activity.—The antibacterial activity of the crystalline substance was tested against a number of bacterial species by the serial dilution method (Table I). The staphylococcus was the most susceptible, followed by the typhoid bacillus and other Gram-negative organisms. The substance therefore differs from gramicidin and penicillin in showing no selective differentiation between Gram-positive and Gram-negative organisms.

TABLE I.—*Inhibition of Bacterial Growth.*

Bacterial species.	Dilution at which inhibitory effect was seen.		
	Complete.	Partial.	None.
1. *Staph. aureus*	160,000	320,000	640,000
2. *S. typhi*	160,000	..	320,000
3. *Bact. coli*	80,000	160,000	320,000
4. *S. Gaertneri*	80,000	..	160,000
5. *Ps. pyocyanea*	40,000	160,000	320,000
6. *Strept. pyogenes*	40,000	..	80,000

TABLE II.—*Inactivation by 10 per cent. of Serum.*

Test organism.	Highest dilution producing complete inhibition.	
	Without serum.	With serum.
Bact. coli	80,000	40,000
Strept. pyogenes	40,000	20,000

Inactivation by serum.—Table II shows the result when 10 per cent. of serum was added in the dilution tests. Greater inactivation was shown on agar plates by the ring test, the substance being dissolved in 50 per cent. serum.

Effect on bacterial respiration.—The oxygen uptake of suspensions of staphylococcus and *Bact. coli* was completely inhibited ten minutes after adding the antibacterial substance to a final concentration of 1 : 1000 (Warburg vessels, 37° C., air).

Tests for Toxicity to Animal Tissues.

Survival of leucocytes.—The method previously described was used (Abraham *et al.*, 1941). A 1 : 800,000 dilution of the substance killed all the leucocytes in one hour; in 1 : 1,600,000 they were sluggish compared with the controls, though they survived for three hours.

Lethal dose for mice.—Three out of five 20 gm. mice which received 0·25 mgm. in 0·2 c.c. of water intravenously died within a few hours; all showed severe distress immediately after the injection. Four which received 0·5 mgm. in 0·2 c.c. of water all died within three hours while 1 mgm. was immediately fatal. When administered by stomach-tube 2·5 mgm. caused death (4 mice), from which it may be inferred that the substance was absorbed from the gastro-intestinal canal. Two mgm. subcutaneously killed in little more than an hour (2 mice). No detailed analysis of the cause of death was made, but 7·5 mgm. injected intravenously into a cat anaesthetized with chloralose caused a sharp rise of blood pressure followed at once by a fall considerably below the initial level.

SUMMARY.

A crystalline substance which inhibits the growth of Gram-positive and Gram-negative organisms has been isolated from the metabolic products of *Penicillium claviforme*. Its action is probably bactericidal. The name claviformin is proposed for it. It is toxic to animal tissues in concentrations which render any therapeutic application unlikely, and moreover its action is inhibited by serum to a considerable extent.

We are indebted to the Rockefeller Foundation for financial assistance in connection with this work.

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OBSERVATIONS UPON SPONTANEOUSLY RECURRING ROUS NO. 1 TUMOURS.

J. G. CARR.

From the Institute of Animal Genetics, University of Edinburgh.

Received for publication June 30, 1942.

THE usual result of the inoculation of Rous No. 1 agent into a fowl is the production of a rapidly-growing, very malignant sarcoma which kills the bird in 3–4 weeks. Several workers have described cases in which the tumours do not grow so rapidly, may remain more or less stationary in size for a considerable period, or regress completely. The general impression is that this is more likely to occur in older birds, and it has been shown that many fowls spontaneously develop antibodies to the Rous No. 1 agent as they grow older (Ledingham and Gye, 1935; Amies, 1937; Duran-Reynals, 1940). Since tumour growth is slower in old birds, this is regarded as evidence of reduced susceptibility.

It has recently been found at this Institute that the suceptibility of healthy 6-week-old Brown Leghorns to inoculation with the Rous No. 1 sarcoma is chiefly determined by the genetic make-up of the individual, and it has been found possible to breed a strain of birds, known as the "Non-Susceptible" or "N-S" strain, which are very resistant to inoculation with the Rous No. 1 agent. The study of this strain is hoped to lead to valuable information as to the mechanics of resistance and susceptibility to neoplasms. An unexpected finding during this work was that Rous tumours appeared in many birds a long time after the inoculation of agent, the original induced tumour (if any) having vanished a considerable time previously. Most workers cannot, for lack of accommodation, keep fowls under observation for extended periods, so that this phenomenon is not likely to have been noticed.

To date 15 instances of recurring tumours have been noted, of which 13 were found in the "N-S" strain. There could be no doubt that these were connected with the inoculation of Rous agent, for spontaneous tumours are very rare in the Institute flock (less than 1 per year). The exact incidence of recurring tumours cannot be stated with any accuracy because of the fluctuating nature of the population, but it is certainly over 10 per cent. The N-S strain are all tested at the age of about 6 weeks by inoculating a dose of Rous agent into the right breast, and successive decimal dilutions of this into the left breast, right leg and left leg respectively. The activity of the agent suspension is simultaneously determined by inoculation in a similar manner

into a group of control birds, and is usually 50–500 minimal infective doses (M.I.Ds.). Most N-S birds grow small tumours at the sites given the largest doses, and these later regress in about 75 per cent. of cases within a period of 28 days. The longest interval found between this test inoculation and the appearance of the delayed tumour is over one year. This case is quoted as an example of the type of finding. Others illustrating various aspects are quoted later.

Fowl L.1833, tested 7.vi.39, dose approximately 1000 M.I.Ds. No tumours detected over a period of 5 weeks. Used for breeding. First egg 18.xi.39, laying and fertility normal, last egg 3.vi.40. Found dead 12.vi.40. At autopsy, typical rapidly-growing Rous sarcomas were found, the largest almost filling the right breast, and successively smaller ones occurring in the left breast and right leg. No tumours were found at any other sites.

In none of these birds have tumours been found at sites other than those inoculated by agent. Though it is extremely unlikely that these tumours could be anything other than Rous No. 1 tumours, it was considered desirable to determine whether or not they contained any Rous agent. The demonstration of agent proved a matter of some difficulty, as the tumours that were discovered by occasional inspection were mostly of the slowly-growing type, from which only a small amount of agent could be anticipated. The rapid tumours usually killed their host before discovery, and were not very common. In 5 cases attempts were made to transmit the tumour, details of which are given later. In these experiments the agent was always prepared by the method of Amies and Carr (1939) (centrifuging a cell-free extract at pH5 on a laboratory centrifuge). Glycerol was used in many cases as a preservative and to ensure that no cells were transmitted. Since there are few data on the rate of loss of activity of Rous agent in 50 per cent. glycerol, the following experiments are submitted in justification of its use in the present work. They are the first three experiments in which the preserving activity of glycerol was quantitatively investigated. The amount of agent in tumours in this paper is given as the least amount of tumour which would yield 1 M.I.D. of agent, as determined by titrating the final product of the processing into 6-week-old susceptible chicks. The activity of a rapidly growing tumour in a young bird lies between 10^{-5} and 10^{-6} g.

EXPERIMENTS ON THE PRESERVING ACTION OF 50 PER CENT. GLYCEROL.

Experiment 1. *Tumour of Fowl* 168.

Processed fresh 2.iv.41. Activity in 2×10^{-5} g. at least.
Part of the tumour preserved in glycerol and processed 14.v.41. Activity in 5×10^{-5} g. at least.

Experiment 2. *Tumour of Fowl* 184.

Processed fresh 26.v.41. Activity in 10^{-6} g.
Part of the tumour preserved in glycerol.
A part of this processed 10.vi.41. Activity in 5×10^{-6} g. at least.
Rest processed 18.vi.41. Activity in 10^{-4} g.

Experiment 3. *Tumour of Fowl* 259.

5.viii.41 : Processed fresh, and agent preserved in 50 per cent. glycerol.
13.viii.41 : Activity corresponded to 5×10^{-6} g. tumour at least.
29.viii.41 : Activity corresponded to 5×10^{-5} g. tumour.
15.ix.41 : Activity corresponded to $2{\cdot}5 \times 10^{-4}$ g. tumour.

It may therefore be assumed that agent could be detected in tumour or suspension preserved by this method if the original tumour contained any recognizable amount of agent. The method of processing enables the final concentration of agent per c.c. to reach any desired value. The usual figure chosen was such that the largest amount in the titration was the equivalent of about 10^{-2} g. tumour. A negative result then indicates that the amount of agent is less than 1/1000 of the amount expected in a routine tumour.

EXPERIMENTS ON THE AGENT CONTENT OF THE RECURRING TUMOURS.

Experiment 1. *Fowl* 0.620.

30.v.41 : Tested, the largest dose containing about 100 M.I.Ds. Small tumours appeared, rapidly regressing.
1.viii.41 : Last inspection before tumour appeared.
20.ix.41 : The right breast was found to be filled with a soft tumour, from the centre of which fluid was frequently tapped.
2.x.41 : Some of this fluid was mixed with an equal volume of glycerol and placed in the cold. Next day it was diluted with 4 volumes of saline and 0·5 c.c. injected into 3 birds. No tumours resulted.
12.x.41 : The bird was found dead. Some of the tumour was placed in 50 per cent. glycerol, and processed for agent 14.x.41. The product was injected in amounts equal to 5×10^{-3} g. into 3 birds. No tumours resulted.
Result.—No agent demonstrated. The type of material used was not particularly suited for its detection.

It has been shown by Andrewes (1931) that tumour exudate may possess strong powers of inactivation towards tumour agents.

Experiment 2. Exact identity of bird unknown, as it was one of two birds in a pen which had lost their identifying wing tabs. It was known to have been tested before 6.v.41.
5.xi.41 : Large tumour found in right breast. The bird was killed, and the tumour found to be white, fibrous, and with a caseous centre. A part of this was transplanted as cells to birds 383, 384, and 385, and gave rise to rapid tumours in all three birds. Another part was preserved in glycerol, and a part immediately processed for agent. This was inoculated into 3 birds at a dose corresponding to 10^{-1} g. tumour. No tumours resulted.
10.xi.41 : The tumour in glycerol was processed for agent, and a dose corresponding to 5×10^{-2} g. tumour inoculated into 3 birds. No tumours resulted. The tumours of birds 384 and 385 were processed for agent, and the yield found by titration to be 10^6 and 10^5 M.I.Ds. per gramme of tumour respectively.
Result.—Agent not present in detectable amounts in the original tumour, but present in normal quantities in transplants.

Experiment 3. *Fowl* 0.274.

6.v.41 : Tested, the largest dose containing about 1000 M.I.Ds. Small tumours appeared in the breasts, but soon regressed.
16.ix.41 : Tumours found in breasts, that on the right being estimated as containing 15 g. material, that on the left less than 0·5 g. Both tumours were found to decrease in size during the next few days.

28.xi.41 : Most of the tumour remaining in the right breast was removed by operation under ether anaesthesia. Two birds were implanted with cells of this material. No tumours resulted. A portion of the tumour was kept in glycerol, and processed for agent 1.xii.41. Amounts corresponding to 2×10^{-2} g. tumour inoculated into 3 birds failed to give rise to any tumours.

29.xi.41 : First egg laid. Further eggs 1.xii and 3.xii.

3.xii.41 : The bird was found to have a severely lacerated comb. This healed easily on treatment, but she was very anaemic and laying ceased. The small tumour in the left breast now began to grow rapidly, and a part of this was removed at biopsy 16.xii.41. Implants into 3 birds failed to produce tumours.

The tumour began to decrease in size again about 10.ii.42 ; laying began again 20.ii.42, and the tumour soon disappeared and has not returned.

Result.—No agent could be demonstrated.

Experiment 4. *Fowl* 0.106.

2.iv.41 : Tested, the largest dose containing about 1000 M.I.Ds. Very small tumours appeared and soon regressed.

16.ix.41 : Tumours found in breasts, that in the right estimated at about 20 g., that in the left about 6 g.

19.ix.41 : First egg laid, followed by 2 more, and then the bird began a partial moult, and the tumours began to decrease in size.

4.xii.41 : Some of the tumour of the right breast was removed under ether. The tumour was very tough. Some was transplanted into 2 birds, but no growth resulted. A portion was preserved in glycerol, and processed next day. Amounts equivalent to $2{\cdot}5 \times 10^{-1}$ g. tumour failed to give rise to any tumours in three birds.

18.xii.41 : The tumour in the left breast had begun to grow very rapidly after the operation, and a portion of this was now removed. This tumour was found to be quite soft. Transplantation of this into 3 birds resulted in all developing very rapid sarcomas. A part of the tumour was processed and the agent preserved in glycerol. This was later injected into 3 birds in amounts corresponding to $2{\cdot}5 \times 10^{-2}$ g. tumour. No tumours resulted. The tumour of one of the transplanted birds was found to contain at least 10^5 M.I.Ds. per gramme of tumour.

3.i.42 : The bird was killed with a large tumour filling the left breast, a tumour half this size in the right breast, and a small tumour in the left leg.

Result.—No agent detected in original tumour, but found in usual amounts in transplants.

Experiment 5. *Fowl* 0.1422.

25.x.41 : Tested, the largest dose containing about 100 M.I.Ds. A single small tumour appeared in the right breast, and rapidly regressed.

26.i.42 : Injected 1·5 mg. methylcholanthrene dissolved in arachis oil into left leg.

7.iii.42 : Injected 5 mg. methylcholanthrene in same way. Similar treatment was given to 4 other N-S birds of about the same age, without the appearance of recurring tumours.

14.iv.42 : Small tumour noted in right breast, and this rapidly enlarged, and by 28.iv.42 filled the whole breast. The bird was then killed, and the tumour processed, the product being inoculated into 2 chicks in amounts equivalent to 10^{-1} g. tumour. No tumours resulted.

Result.—No agent demonstrated.

A portion of all the tumours removed from the last four birds with recurring tumours and all tumours resulting from cell implants or inoculation with agent were examined histologically. In all cases the appearance was that of a Rous No. 1 sarcoma. When a negative result followed inoculation of cells or agent preparation from these recurring tumours into other birds, one or more of the group was later inoculated with corresponding material from a routine Rous tumour. In all cases the birds were thus proved to have been susceptible.

It thus appears that the transplants of the recurring tumours are typical in every way. One line of tumour originating in this way from Expt. 2 was carried for three further generations by agent, and was indistinguishable from the routine Rous tumour in every respect. The apparent absence of the agent in the original recurring tumours is notable. The fact that in most cases the tumour was growing rather slowly and in an old bird may partially explain the failure to find any agent, but the point seems to call for further investigation.

TRANSPLANTABILITY OF THE RECURRING TUMOURS IN N-S BIRDS.

These recurring tumours may have appeared either as a result of a decrease in the susceptibility of the host, or as the result of the mutation of either a tumour cell or an agent particle to a form which would grow more readily in the host. The absence of detectable agent, and the appearance of the tumour at more than one inoculation site, do not favour the latter hypothesis. If such a change had occurred in the tumour, it is reasonable to expect that the tumour would grow also in ordinary N-S birds. Attempts to obtain growth of either the cells of the recurring tumours, or grafts of them growing in normal chicks, or of agent preparations of either of these, by inoculating them into N-S birds whose agent-induced tumours had regressed, were never successful.

The alteration which elicits the formation of the tumour is therefore presumably in the host and not in the tumour.

EFFECT OF LAYING ON THE GROWTH OF RECURRING TUMOURS.

The suggestion in the case of 0.274, in Expt. 3, that egg production and tumour growth are antagonistic has been noticed in several instances. Another case may be briefly mentioned as having some interesting features.

Bird 0.135. 22.iv.41. Tested, largest dose containing about 50 M.I.Ds. Small tumours soon regressed. Laying 7.iii.41 to 3.x.41, then stopped. Small tumour found in right breast 20.xii.41. Laying began again 1.i.42, and by 10.i.42 no tumour was detectable.

20.i.42 : Comb was injured by bird in neighbouring cage. Laying ceased, and the tumour began to grow again. This growth never reached a large size, and later regressed. Laying recommenced on 23.ii.42, and the bird has remained normal.

This is not an invariable finding, however, for there have been two cases in which the bird has continued to lay in spite of a recurring tumour found at a periodic inspection.

TRAUMA IN RELATION TO INDUCTION OF RECURRING TUMOURS.

As will be noted in the case histories quoted, there are occasions in which the recurring tumour is prefaced by a history of injury, due either to accident or operation. There are two other examples in the present series. One may be briefly mentioned.

Bird 0.311. Tested 27.v.41, the largest dose containing about 1000 M.I.Ds. 1.vii.41 : Skin on top of head badly torn, presumably by the other birds in the pen. Healing was rapid, but tumours were seen to be developing a few days later, and the bird was killed 8.viii.41, and found to have several nodules of tumour in the right breast, nothing in the left breast, a very large tumour in the right leg, and nothing in the left leg. There was no indication of tumour formation at the site of the head injury.

Stimulation of tumour growth as a result of injury has not been observed in young birds. On several occasions the N-S birds have been injured by their cage-mates while being tested, but this has never resulted in any increased growth of tumour. Also, there have been some cases of accidental injury to mature N-S birds without any tumour growth resulting. This connection between tumour recurrence and injury is therefore no more than suggestive.

SEX INCIDENCE IN N-S BIRDS.

All 13 cases of recurring tumours in the N-S strain have been found in females. No significance can be attached to this, however, as only a very few males are retained for breeding purposes.

BIRDS OTHER THAN N-S STRAINS.

There have been two other examples of this delayed growth of tumours in birds from other strains of the Institute stock. One was a male which had grown very small tumours which soon regressed, and the other was a male which produced no tumours at all in response to a large dose of agent. Each bird was found with large progressive tumours about two months after inoculation.

DISCUSSION.

The frequency with which this recurrence has been found is certainly surprising. It appears to occur in birds at about the time that the early rapid growth is becoming reduced, and usually also in non-layers. As suggested above, the cause is most likely to be a decrease in the resistance of the host. Such a decrease of resistance to tumour growth with age is contrary to many of the findings of the workers who use mammalian transplanted tumours. But the two cases are not at all comparable, since the conditions governing tumour incidence and host resistance in this work are quite different from those encountered in most other types of experimental tumour research. Although many experimenters have shown (Ledingham, 1935 ; Amies, 1937 ; Duran-Reynals, 1940) that old birds develop serum antibodies to Rous agent as they age, and that tumours grow more slowly in older birds, neither of these facts are proofs of increased resistance, for such antibodies have no effect upon the growth of tumour cells, and the reduced rate of growth may well be due merely to the reduced rate of vital processes in old animals.

It was possible to test this hypothesis on a limited scale by re-inoculating with Rous agent 4 tested N-S males one year after the test inoculation. In all birds the tumours were found to reach a much larger relative size and to regress

very much more slowly than at the first test. In one case the tumour grew until it killed the host. The tumours certainly grew rather more slowly than in young chicks, but the fact that any growth at all took place in animals which had received the equivalent of an immunizing dose indicated that a marked reduction in resistance had indeed occurred.

The chief objection to such a theory lies in the fact that only a proportion of the birds developed the recurring tumours, whereas according to this supposition, the resistance of all birds will decrease with age. Either in some birds the cell or agent responsible for the tumour did not survive at all, or the degree to which the resistance drops is not sufficient for tumour growth to occur in many birds. A variation in this decrease of resistance is certainly indicated by the variation in the time between inoculation and the development of the recurring tumour. It may also be that some stimulus is required in addition to provoke tumour development. The relation of trauma to the development of the tumours makes it possible that this may act either by providing a direct stimulation for tumour growth, or by reducing the resistance of the host to tumour development. Such a stimulation of a latent tumour bears a close resemblance to the findings of MacKenzie and Rous (1941) on the effect of wounds on tar lesions in rabbits. It is notable that all the recurring tumours (except the methylcholanthrene-treated 0.1422) were found in birds running together in a pen, where trivial injuries due to fighting, etc., are common, and never in those birds kept in individual cages, where such injuries are rare. Such stimulation as a result of injury is confined to the older birds, however.

Since the tumours appeared only at the site of injection, it must be presumed that tumour cells or agent were immobilized and preserved at these points. The absence of tumours at other points suggests that any agent which may have been disseminated during the growth of the first tumour (Mellanby, 1938) either was not thus preserved, or was no more capable of inducing a tumour at a later time than at the beginning. It is difficult to imagine that the agent which remained at the site of injection could have any other than an intracellular existence. If this cellular infection produced a neoplasm, as one would expect, it must have been exceedingly small in size, for post-mortem examination of a large number of birds whose tumours had regressed has never revealed anything which could be regarded as a latent tumour ; the regression has always been complete, as far as could be judged by macroscopical examination. In addition, since the relative sizes of multiple tumours often approximated to the size expected from a consideration of the dose of agent originally given, more than accidental survival of a single agent particle or cell is involved, and it becomes even more difficult to obtain a clear idea of the conditions which govern such a survival.

A further consideration of this matter is best left until some data as to the cause of the resistance of these birds to the inoculation of Rous agent becomes available. It is to be hoped that this will also shed some light on the mysterious apparent absence of the agent in these tumours, and the equally strange reappearance of it in cell grafts into other birds. Though the sudden appearance of a non-filterable phase of Rous tumour is well recognized, this has usually been of a capricious type. In the present work this state of affairs

regularly follows a certain procedure. The failure of the agent preparations to produce tumours cannot be due to the presence of inhibitory substances of the type which has often been reported in filtrates of the Rous No. 1 sarcoma by several workers (Gye and Purdy, 1931 ; Sittenfield, Johnson and Jobling, 1931 ; Murphy and Sturm, 1932), since the agent was always separated from the extract by the method of processing which was here employed, and the inhibitor should have remained behind. The only factor which differs from the normal seems to be the age of the birds. Unfortunately present conditions make it impossible to carry out any work requiring a number of old birds, so that both this suggestion, and the suggestion that older birds are more susceptible, cannot be directly tested.

Finally there can be no doubt that our ideas as to the range of action of the classical tumour-inducing viruses must be considerably extended.

SUMMARY.

A number of cases are described of Rous No. 1 tumours appearing many months after inoculation of the agent into birds and regression of any tumours thus induced. This is believed to be due to a reduction in the resistance of the host. Agent could not be demonstrated in such tumours, but was present in the usual amounts in grafts from them.

Acknowledgments.—My thanks are due to Mr. K. Chodnik for assistance with this work. All expenses in connection with this work were borne by the British Empire Cancer Campaign.

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INTERFERENCE BY ONE VIRUS WITH THE GROWTH OF ANOTHER IN TISSUE-CULTURE.

C. H. ANDREWES.

National Institute for Medical Research, Hampstead, London, N.W. 3.

Received for publication July 3, 1942.

A NUMBER of instances are on record in which one virus interferes with the pathogenic action of another in the infected host. The two viruses concerned may be two strains of one virus, for example the neurotropic and viscerotropic strains of yellow fever studied by Hoskins (1935); or they may be serologically unrelated as are the viruses of yellow fever and Rift Valley fever (Findlay and MacCallum, 1937). As a rule only one of the viruses concerned, possibly that which multiplies or spreads more rapidly, has an interfering effect and the relation is not a reciprocal one. On the other hand, two or more viruses may simultaneously infect and damage the same cell, as can be shown by finding several types of inclusion body in one cell (Syverton and Berry, 1936). Again, these two types of behaviour may occur with the same virus combinations; virus III and Shope fibroma viruses can simultaneously produce changes in cells of rabbits, but if virus III is on the spot first, it may suppress the action of the fibroma virus (Andrewes, 1940). The interference phenomenon may be seen when two viruses are inoculated together, or when one is inoculated ahead of the other.

I have tried to study the interactions of viruses in tissue-cultures, using the W.S. strain of Influenza A virus and its neurotropic variant (Stuart-Harris, 1939), known in our laboratory as "neuroflu."

TECHNIQUE.

Cultivation was carried out by a modified Maitland technique. Minced embryo, about 0·1 gm. of 11-or 12-day-old chick embryos (without eyes, beak or legs) was placed in Carrel flasks with 4 c.c. Tyrode's solution, and the virus inoculum consisted of 0·25 c.c. of the fluid from a previous culture. Flasks were incubated at 37° C. Transfers were made at 3- or 4-day intervals; cultures could be stored at −2° C. for a few weeks when continuous passage was unnecessary. Both strains were readily propagated by this technique, and had been carried through five tissue-cultures at the beginning of the experiments.

The presence of W.S. virus was shown by the presence of consolidation in the lungs of intranasally inoculated mice which were killed after 6 days, or

died before that time. Neuroflu infection was considered to be present when mice died with extended legs 3–12 days after intracerebral inoculation. Commonly symptoms as described by Stuart-Harris were present for a day or two before death, but mice looking well one day were sometimes found dead next morning.

The supernatant fluid of centrifuged tissue-cultures of the W.S. strain would infect mice by the intranasal route in dilutions of 1 : 10^3 or 1 : 10^4, but produced no symptoms on intracerebral injection. The neuroflu virus killed mice when given by the intracerebral route in dilutions up to 1 : 10^4 in earlier trials ; later it was as a rule not lethal in higher dilutions than 1 : 100. Undiluted cultures of neuroflu, when given by the intranasal route, produced pneumonia which was fatal in some mice, but higher dilutions caused no lesions, or trifling ones.

RESULTS.

In the experiments to be described, flasks were inoculated with one virus and experiments carried out to determine whether such infected cultures would permit survival or growth of the other virus added later. A series of flasks was prepared with Tyrode's solution and minced embryo, and to half was added 0·25 c.c. W.S. culture-virus. All were incubated for 24 hours. Then each received 0·25 c.c. of centrifuged neuroflu culture. Four days later films were stained to test for bacterial contamination and, the films being satisfactory, the various cultures were tested by intracerebral inoculation of 0·02 c.c. of supernatant fluid to each of a group of three mice. The result is shown in Table I.

TABLE I.—*Suppression of Neuroflu in Cultures Previously Infected with W.S.*

Cultures receiving :							
W.S. followed by neuroflu.				Neuroflu only.			
A.	B.	C.		D.	E.	F.	G.
S	S	†6	.	†5	†4	†5	†5
S	S	S	.	†5	†5	†5	†5
S	S	S	.	†5	†5	†5	†5

The vertical columns show the results of testing seven separate cultures intracerebrally on groups of three mice.

S = mouse survived 14 days.
†5 indicates death after 5 days, etc.

It will be seen that amongst 9 mice receiving cultures A, B, C, inoculated first with W.S., then with neuroflu, only one death occurred ; W.S. does not produce symptoms when injected intracerebrally, and growth of neuroflu failed, or almost failed to occur in cultures in which W.S. was growing. Without the W.S. the neuroflu grew well and killed all the 12 test mice. The results are satisfactorily sharp because influenza virus does not survive at 37° C. in the absence of susceptible living cells ; hence neuroflu which failed to multiply, failed also to survive.

This experiment was carried out with essentially the same results eleven times in all. In four experiments all the mice receiving the doubly infected cultures survived, while all controls died ; in four tests the results were almost exactly as in Exp. 1 ; in two they were a little less clear-cut. Light infections with W.S. virus (one-twenty-fifth of the usual) failed to interfere wholly with the growth of neuroflu even when the latter was correspondingly reduced in quantity ; nor was any interference shown by undiluted W.S. virus in the form of mouse-lung filtrate unadapted to growth in tissue culture ; in effect this probably acted much like a small dose of culture-adapted virus. Neuroflu could grow normally in cultures in which the Tyrode's solution was replaced by virus-free ultrafiltrate of a 24-hour W.S. culture. This indicates that no soluble substance inhibiting virus growth was present in the fluid part of the cultures.

Factors of Time and Temperature.

When cultures infected with W.S. were held for shorter periods—10 minutes and 6 hours at 37° C.—before adding the neuroflu, the latter was able to grow ; but the results were not absolutely sharp since 4 out of 15 mice inoculated with these cultures survived, whereas 12 out of 12 controls injected with neuroflu only died. Similarly when W.S. cultures were held at —2° C. or 20° C. before adding the neuroflu, the latter was able to multiply ; again results were not perfectly sharp ; 12 of 12 mice receiving neuroflu alone died, while 8 of 21 survived when contact between W.S. and cells was 24 hours at —2° C., and 1 of 9 survived when contact was at 20° C. It nevertheless appears that the W.S. virus had to be given a good opportunity for multiplication before it could regularly render the medium unable to support the growth of the neuroflu. The lack of sharpness in the results can probably be explained by the facts recorded in the next paragraph.

Infection of Cultures with Two Viruses Simultaneously.

In an early experiment, in 1939, a mixture of undiluted W.S. culture with a 1 in 10 dilution of neuroflu was added to two cultures, while two others received 1 in 20 neuroflu only. No interference occurred ; neuroflu was present 4 days later in all cultures in dilutions from 1 in 10 to 1 in 10^4. Experiments performed two years later gave different results, probably because the neuroflu was not then attaining such high titres in cultures, while the W.S. still was active in high dilution (see Table II). Cultures were inoculated with mixtures in equal parts of undiluted W.S. culture and neuroflu undiluted 1 in 10 and 1 in 100. Control flasks received neuroflu alone diluted as shown.

TABLE II.—*Suppression of Neuroflu (NF) by W.S. Inoculated along with it.*

W.S. + NF.			W.S. + NF/10.			W.S. + NF/100.			NF/2.			NF/20.			NF/200.		
Culture:																	
A	B		C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
†7	S	.	S	S	S	S	S	S	†3	†4	†3	†3	†3	†5	S	S	S
S	S	.	S	S	S	S	S	S	†4	†5	†3	†3	†3	†6	S	S	S
S	S	.	S	S	S	S	S	S	†6	†7	†3	†4	†4	†6	S	S	S

Legend as in Table I except that S indicates survival for 10 days (when the experiment was ended).

Table II shows that cultures receiving neuroflu 1 in 20 all "took," but that no virus was detected in those infected with a tenth of that dose. Mixture with an equal volume of W.S. virus sufficed (cultures A to E) to suppress almost wholly the neuroflu, but it will be seen that this interference was effective against very small quantities of neuroflu. In the earlier experiment 10 to 100 times as much neuroflu virus had been present in the mixture.

Interference by Neuroflu with Growth of W.S.

The phenomena described cannot be explained by a more rapid growth on the part of the W.S. virus, for it could be shown that under appropriate conditions the neuroflu in its turn could interfere with the growth of the W.S. Unlike the W.S., which without long adaptation will not kill mice by the intracerebral route, the neuroflu was not innocuous to the mouse's lungs. A more elaborate quantitative technique had therefore to be used.

Flasks were infected with neuroflu and a 1 in 10 dilution of W.S. virus added 24 hours later. Four days later cultures were titrated as shown in Table III, both intranasally and intracerebrally.

TABLE III.—*Suppression of W.S. in Cultures Previously Infected with Neuroflu.*

	NF only	NF before W.S.				W.S. only					
	I.N.	I.N.	I.C.	I.N.	I.C.	I.N.			I.N.		
Undiluted	. ..	..	†4 †4 †6	..	†6 †6 †7	..			..		
1 in 10	. + 0 0	+ + 0	..	0 0 0	..	..			..		
1 in 100	. 0 0 0	+ + 0 0	..	0 0 0	..	†5	++	++	++	++	+
1 in 1000	. 0 0 0	0 0 0	..	0 0 0	..	†5	++	++	++	+	+
1 in 10^4	. ..	0 0 0	..	0 0 0	..	++	+	+	+	+	0

Legend as in Table I: ++ and + signs indicate degrees of consolidation of mouse lungs.
I.N. = Intranasal inoculation.
I.C. = Intracerebral ,,

Cultures receiving W.S. only infected mouse-lungs at a dilution of 1 in 10^4, while those which had previously received neuroflu contained less than a hundredth as much W.S. virus, and possibly none at all, because neuroflu alone can produce lung-lesions in low dilutions. A repetition of this experiment produced a similar result. It thus appears that each of the W.S. and neuroflu viruses has the power to suppress the other if it is first in the field.

Specificity of the Interference in Cultures.

(i) *P.R. 8 and Neuroflu.*

One experiment was carried out with the P.R. 8 strain of Influenza A. This is serologically distinct from W.S. though there is some antigenic overlap. Cultures of this were incubated for 24 hours and neuroflu, which is serologically identical with W.S., was then added. Its growth was wholly suppressed, as in the earlier tests.

(ii) *Lymphogranuloma venereum and W.S.*

This virus was grown by the same technique as for influenza. Minor modifications, including corking of the Carrel flasks, were desirable for continued propagation of the lymphogranuloma virus, but for short experiments the medium used for influenza cultures was satisfactory. A paper dealing with the cultivation of lymphogranuloma virus will be published shortly. Cultures containing lymphogranuloma virus were tested by intracerebral inoculation of mice ; they produced characteristic symptoms of infection, but did not kill regularly. Such tests were of course practicable even in the presence of W.S. virus. Though lymphogranuloma virus will produce lung lesions when inoculated intranasally into mice, the cultures in this series did not contain enough virus to do so ; at most tiny lesions were seen in an occasional mouse lung. Intranasal inoculation could thus be used to test for the presence of W.S. virus in the presence of lymphogranuloma. In one series of tests W.S. virus was added to half of a series of cultures and lymphogranuloma to all 24 hours later. Intracerebral tests in mice showed that the lymphogranuloma grew just as readily in the W.S. infected cultures as in controls. In the complementary experiment, W.S. virus was similarly added to control flasks and to some in which lymphogranuloma had grown for 24 hours ; the W.S. grew equally well in all cultures. There was thus no evidence that either of these viruses could interfere with the growth of the other.

(iii) *Fowl-pox and Neuroflu.*

A strain of fowl-pox was grown in chick embryo cultures in Tyrode's solution. Neuroflu added to such cultures after 24 hours grew as well as in controls. The activity of the fowl-pox in the cultures was proved by rubbing into breasts of pigeons from which some feathers had been plucked.

(iv) *Vaccinia and W.S.*

Similar experiments were carried out with a strain of vaccinia (Lister lapine) and influenza (W.S.), both viruses being carried in chick embryo-Tyrode cultures in Carrel flasks. Tests of all cultures for W.S. could be made by intranasal inoculation of mice, since this strain of vaccinia produced no lung lesions when thus tested. The vaccinia was estimated by intradermal titration in shaved rabbits' skins. Previous infection of cultures with vaccinia had no obvious effect on the ability of the W.S. to multiply. The converse experiment gave discordant results. From one experiment it appeared that vaccinia grew very poorly in W.S. treated cultures as compared with controls. This result was not repeated in later tests, but interpretation was more difficult than in the experiments with other viruses, since vaccinia will survive well at 37° C. for some days in the absence of cells ; clear-cut results could not therefore be expected.

(v) *Vaccinia and Lymphogranuloma Venereum.*

Infection of cultures with lymphogranuloma did not appear to affect the growth of vaccinia, though, because of the relative stability of vaccinia at

37° C., this result must be accepted with caution. The converse experiment is impracticable as both viruses infect mice intracerebrally.

Much more work could profitably have been carried out by the technique described on relationships between different viruses in culture; but further experiments have had to be deferred through pressure of other work.

DISCUSSION.

One fact stands out clearly from the experiments described: When a strain of Influenza A is given good opportunity to multiply in a tissue culture of the Maitland type, that culture is thereby rendered incapable of supporting the growth of another strain of influenza added subsequently. This can not be because of any formation of antibody, nor is there any change in pH as compared with uninoculated cultures. Ultrafiltrates of infected cultures form an adequate medium in which to obtain growth of newly added virus when fresh cells are also added. The cells are presumably altered, though they can scarcely be killed, as they will still support the growth of another virus which needs living cells in which to multiply. The most obvious explanation of the phenomenon is probably the correct one—that the virus first upon the scene uses up some essential foodstuff in the cells. Consumption of such a metabolite rather than an actual "killing" of cells is very possibly why, when a fairly heavy influenza virus inoculum is added to cells in a Maitland culture, no further multiplication occurs after 24 to 48 hours. In cultures where physiological conditions are more closely imitated, as with the roller-tube technique, viruses may continue to multiply for long periods (Gey and Bang, 1939). It is quite possible that in such cultures the postulated foodstuff could be regenerated and that no "*in vitro*" interference phenomenon would occur. An alternative to the hypothesis of an exhaustion of food-supply would be, of course, the generation within the cell of some poorly diffusible inhibitory substance.

The interference between two strains of influenza virus suggests that these viruses attack the same cells in chick embryo. Failure of two viruses to interfere with each other, for instance influenza and lymphogranuloma venereum, might be explained by a biochemical difference in their demands upon a cell for food or by a difference in the type of cell attacked. Studies in cultures of one type of cell would be necessary in order to decide the cause of a lack of interference between two viruses.

The reason is harder to determine for the interference between two influenza strains inoculated simultaneously but in different proportions. Consideration of the rate of increase of influenza virus in tissue culture shows that, with a heavy inoculum (0·25 c.c. of active culture virus to 4 c.c. of medium), the multiplication is only about 16-fold; enough, in fact, to cancel out the effect of dilution and restore the titre to the level of the inoculated virus; the maximum is reached in about 24 hours. With a light inoculum (one-thousandth of the other), little rise may be demonstrable in 24 hours, but the same maximum titre may be reached after 48 hours, as with the heavy inoculum. Thus, if one infects a culture with much of W.S. virus and very little of neuroflu,

conditions for virus-growth as a whole will presumably have become unfavourable before the neuroflu has got fairly started. In practice the interference between simultaneously inoculated viruses was only seen when the one inoculated in smaller quantity was so scanty that it could not certainly be detected unless and until it had had a chance to multiply. Thus its growth may not have been wholly suppressed, but only kept below the level at which it could be recognized. It seems likely that the results recorded would be obtained only if the virus particles added to a culture were very numerous relatively to the numbers of susceptible cells; one could then consider the happenings in any one cell as comparable with what was occurring in the culture as a whole.

I have used the terms "interference" and "*in vitro* interference phenomenon," but do not wish to imply that the *in vivo* interference of Hoskins and others is necessarily to be explained along the lines discussed above. It would, however, be of interest to see whether there were a close parallelism between interference of two viruses with each other *in vivo* and *in vitro*. The method of study seems to offer possibilities for approaching the difficult problem of virus-metabolism, or at any rate for discovering how viruses resemble or differ from each other as regards their metabolic needs.

SUMMARY.

Cultivation of Influenza A virus in a simple tissue-culture rendered the culture unable to support the growth of a biologically distinct strain of Influenza A virus when this was added 24 hours later.

Such an altered tissue-culture was still able to allow multiplication of an unrelated virus such as that of lymphogranuloma venereum.

Whichever strain of influenza virus was added to the culture first could interfere with the growth of the other.

When two strains of Influenza A virus were added to a tissue-culture simultaneously, but in widely differing amounts, the one present in larger quantity suppressed the growth of the other.

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